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## Heat-Induced Perturbations during Oocyte Maturation Carry Over to Increase Susceptibility of Preattachment Embryos to Heat Stress

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To the Graduate Council:

I am submitting herewith a thesis written by Amber Nicole Bogart entitled "Heat-Induced Perturbations during Oocyte Maturation Carry Over to Increase Susceptibility of Preattachment Embryos to Heat Stress." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

J. Lannett Edwards, Major Professor

We have read this thesis and recommend its acceptance:

Frank Neal Schrick, Michael O. Smith

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Over to Increase Susceptibility of Preattachment Embryos to  
Heat Stress**

A Thesis Presented for the Master of Science Degree

The University of Tennessee, Knoxville

Amber Nicole Bogart  
December 2007

## **Dedication**

This thesis is dedicated to my loving parents Diana and John Bogart. Without their unconditional love and support, I would not have been able to get to where I am today. Thank you for always encouraging me to pursue my dreams no matter what obstacles may lay ahead. I hope that I have made you proud.

## **Acknowledgments**

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Last but not least, I would like to thank my family who was there for me through my endless frustrations. Matt, to you I owe everything. Thank you for your love, support and patience. I could not ask for a better person to share this time.

## Abstract

Objectives were to 1) examine effects of heat stress on maturing oocytes to alter the ability of resulting embryos to develop after fertilization, 2) evaluate blastocyst development of compact morulae derived from control or heat-stressed oocytes after culture at 38.5°C or 41.0°C, and 3) evaluate effects of heat stress on compact morulae to alter sex ratio. Culture of cumulus oocytes at 41.0°C did not alter ability of presumptive zygotes (**PZ**) to cleave; however, the proportion of embryos that cleaved to the 8- to 16-cell stage was lower ( $P = 0.01$ ). Also, the proportion of 8- to 16-cell embryos derived from heat-stressed oocytes undergoing compaction was lower ( $P = 0.01$ ). Effects of heat stress to reduce compaction was without consequence on morphology, as quality scores of embryos derived from heat-stressed oocytes were similar to those derived from nonheat-stressed oocytes. Application of heat stress to compact morulae derived from nonheat-stressed oocytes did not affect blastocyst development. Also, blastocyst development of compact morulae derived from heat-stressed oocytes was similar to those derived from nonheat-stressed controls. However, if compact morulae derived from heat-stressed oocytes were exposed to 41.0°C, blastocyst development was reduced ( $P = 0.007$ ). This effect was coincident with an increase in the proportion of degenerate embryos ( $P = 0.02$ ). Temperature at which oocytes were matured did not alter sex ratio of resulting blastocysts. Rather, there was a tendency for embryo temperature to increase the proportion of male embryos that survived ( $P < 0.06$ ). Results described herein are

significant as they not only clarify the extent to which heat stress during maturation reduces embryonic development after fertilization but are also informative of heat-induced perturbations that carry over to increase the susceptibility of resulting embryos to heat stress. With this in mind, results raise possible concerns about current practices of utilizing MOET in heat-stressed cattle with the intent of obtaining “developmentally-competent” embryos. Additionally, since it was also demonstrated that developmental competence of oocytes has an impact on the responsiveness of resulting embryos to heat stress, effort to clarify developmental competence of oocytes before utilizing in vitro derived embryos to improve fertility of heat-stressed dairy cows is needed. .



## Nomenclature

d	days
°C	degrees Celsius
°F	degrees Fahrenheit
h	hour(s)
µg	microgram
µl	microliter
µM	micromolar
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar

## Abbreviations

AI	anaphase I
AQP	aquaporin
Ca <sup>2+</sup>	calcium
CO <sub>2</sub>	carbon dioxide
Cl <sup>-</sup>	chloride
Cx43	connexin 43
CG	cortical granules
COCs	cumulus oocyte complexes
cAMP	cyclic adenosine monophosphate
DNA	deoxyribonucleic acid
Dsc	desmocollins
Dsg	desmogleins
EGA	embryonic genome activation
FBS	fetal bovine serum
FSH	follicle stimulating hormone
G1	gap phase 1
G2	gap phase 2
GV	germinal vesicle
GVBD	germinal vesicle breakdown
GSH	glutathione
G6PD	glucose-6-phosphate dehydrogenase
GnRH	gonadotropin-releasing hormone
hCG	human chorionic gonadotrophin
HEPES	N-2-hydroxyethyl piperazine-N'-ethanesulfonic acid
hIVM	hours of in vitro maturation
hpi	hours post insemination
hpm	hours post maturation
HSP	heat shock proteins
ICM	inner cell mass

IVF	in vitro fertilization
IVM	in vitro maturation
K <sup>+</sup>	potassium
LH	luteinizing hormone
MPF	maturation promoting factor
M199	medium-199
mRNA	messenger ribonucleic acid
MI	metaphase I
MII	metaphase II
MAPK	mitogen activated protein kinase
mKSOM	modified potassium simplex optimized medium
MOET	multiple ovulation embryo transfer
N <sub>2</sub>	nitrogen
NTC	no template control
Na <sup>2+</sup>	sodium
OMM	oocyte maturation medium
O <sub>2</sub>	oxygen
Poly(A)	polyadenylated
PVA	polyvinyl alcohol
PZ	presumptive zygote
P <sub>4</sub>	progesterone
PN	pronuclei
PKC	protein kinase C
RNA	ribonucleic acid
RER	rough endoplasmic reticulum
SER	smooth endoplasmic reticulum
SEM	standard error of the mean
TI	telophase I
TII	telophase II
TALP	Tyrode's Albumin Lactate Pyruvate
ZP	zona pellucida
ZO	zonula occludens

## Table of Contents

<b>Chapter 1 Introduction .....</b>	<b>1</b>
<b>Chapter 2 Literature Review .....</b>	<b>5</b>
<b>The Making of a Blastocyst .....</b>	<b>5</b>
<b>Oocyte Maturation .....</b>	<b>5</b>
Nuclear Maturation.....	7
Cytoplasmic Maturation .....	9
<b>Fertilization .....</b>	<b>12</b>
<b>Cleavage of the Early Embryo .....</b>	<b>14</b>
<b>Embryonic Genome Activation .....</b>	<b>16</b>
<b>Compaction and the Role of E-Cadherin .....</b>	<b>17</b>
Blastomere Polarization within the Preattachment Embryo .....	19
Formation of Gap Junctions in the Preattachment Embryo.....	20
Formation of Tight Junctions in the Preattachment Embryo .....	23
<b>Formation of the Blastocyst-Stage Embryo .....</b>	<b>24</b>
Na <sup>+</sup> K <sup>+</sup> ATPase Activity during Blastocoele Formation.....	25
Aquaporins in Blastocoele Formation .....	26
Desmosomes .....	27
<b>Heat Stress and its Impact on Reproduction in the Dairy Cow .....</b>	<b>28</b>
<b>Effects of Heat Stress during Estrus to Decrease Fertility .....</b>	<b>30</b>
<b>In Vitro Model to Study Direct Effects of Heat Stress on the Maturing Oocyte .....</b>	<b>32</b>
<b>Direct Effects of Heat Stress on the Maturing Oocyte .....</b>	<b>34</b>
<b>Effects of Heat Stress on the Preattachment Embryo.....</b>	<b>39</b>
<b>Possible Heat-Induced Alterations in Sex Ratio of Preattachment Embryos .....</b>	<b>41</b>
<b>Effects of Heat Stress on the Maturing Oocyte to Possibly Carry Over into Preattachment Development to Increase Susceptibility of Compact Morulae to Heat Stress .....</b>	<b>43</b>

<b>Chapter 3 Materials and Methods .....</b>	<b>45</b>
Materials .....	45
In Vitro Production of Embryos .....	45
Heat Induced Perturbations during Oocyte Maturation Carry Over to Increase Susceptibility of Compact Morulae to Heat Stress.....	46
Sex Determination of Embryos .....	48
<b>Statistical Analyses .....</b>	<b>53</b>
<b>Chapter 4 Results and Discussion.....</b>	<b>55</b>
<b>Conclusions/Implications .....</b>	<b>68</b>
<b>List of References .....</b>	<b>69</b>
<b>Vita.....</b>	<b>99</b>

## **List of Figures**

Figure 1. The making of a blastocyst-stage embryo. ....	6
Figure 2. Experimental design and developmental endpoints. ....	47
Figure 3. Dissociation peaks observed in male and female embryos and in no template controls (NTC).....	52
Figure 4. Blastocyst development of heat-stressed or nonheat-stressed compact morulae derived from COCs undergoing IVM at 38.5 or 41.0°C.....	61
Figure 5. Blastocyst development of compact morulae derived from heat-stressed or nonheat-stressed but otherwise "developmentally challenged" oocytes following culture at 38.5 or 41.0°C.....	64

# **Chapter 1**

## **Introduction**

Heat stress is a costly problem to the dairy industry, as high environmental temperatures and relative humidity decrease milk yield (reviewed by West, 2003) and fertility (reviewed by Hansen, 2007). Reduced fertility is in large part attributable to elevations in body temperature. Ulberg and Burfening (1967) observed an approximate 25% decrease in pregnancy rate for every 1°C elevation in rectal temperature.

The effect of heat stress to reduce fertility are most problematic during estrus (i.e., when the oocyte is maturing within the ovulatory follicle in preparation for fertilization). Increased ambient temperatures (Stott and Williams, 1962) and heat-induced elevations in uterine and rectal temperatures (Gwazdauskas et al., 1973) on the day of breeding have been negatively correlated with conception rates. Cooling lactating dairy cows prior to the day of breeding, improves pregnancy rates but is not entirely effective for increasing pregnancy rates to those observed during nonheat-stressed conditions (reviewed by Hansen and Aréchiga, 1999).

Heat-induced reductions in fertility are likely due to direct effects of elevated maternal temperature to compromise the maturing oocyte. Putney et al. (1989) showed that increases in body temperature  $\geq 41.0^{\circ}\text{C}$  during the first 10 h of estrus reduced the proportion of good to excellent quality embryos and increased the number of retarded or degenerate embryos recovered 7 days after

artificial insemination in superovulated heifers. Furthermore, culture of maturing oocytes at 41.0°C (a physiologically-relevant elevated temperature; Monty and Wolff, 1974, Roman-Ponce et al., 1977; Ealy et al., 1993), decreases embryonic development after in vitro fertilization (**IVF**) by 42 to 65% (Edwards and Hansen, 1996; Lawrence et al., 2004; Roth and Hansen, 2004; Edwards et al., 2005; Schrock et al., 2007).

Effects of heat stress to reduce developmental competence of maturing oocytes are dependant on the duration and severity of elevated temperature (Edwards and Hansen, 1996) and are coincident with heat-induced perturbations occurring in the ooplasm. Possibilities include increased glutathione content (Payton et al., 2003), decreased synthesis of intracellular proteins (Edwards and Hansen, 1996, 1997), and decreased abundance of polyadenylated mRNA (Payton and Edwards, 2005). Moreover, Edwards et al. (2005) reported that heat-stressed oocytes matured faster than nonheat-stressed oocytes. Specifically, oocytes matured at 41.0°C resulted in more oocytes at MI and MII by 8 and 18 h after placement in maturation medium. An effect of hastened maturation is not without consequence, as insemination of heat-stressed oocytes at the same time as nonheat-stressed controls likely results in the fertilization of an “aged” oocyte. In support of this, earlier fertilization was beneficial for improving development of heat-stressed oocytes (Edwards et al., 2005; Schrock et al., 2007).

Regardless of specific mechanism, it is important to note that the effects of heat stress to reduce oocyte development after fertilization are not “all or none”.

Some heat-stressed oocytes do develop to the blastocyst stage and are morphologically equivalent in both stage (early, normal, expanded, or hatched blastocyst) and quality compared to those derived from nonheat-stressed oocytes (Schrock et al., 2007). However, because blastocyst formation is not indicative of an embryo's ability to develop further (i.e., establish or maintain a pregnancy), further inference is limited.

Like the maturing oocyte, early cleavage-stage embryos are susceptible to effects of heat stress. Two-cell embryos are particularly susceptible, as heat stress decreases development by 31 to 100% compared to nonheat-stressed controls (Ealy et al., 1993, 1995; Edwards and Hansen, 1997; Rivera and Hansen, 2001). As embryos proceed in development however, ability to withstand elevations in temperature increases (i.e., they become more thermotolerant; Ealy et al., 1993, 1995; Edwards and Hansen, 1997). This is true regardless if embryos are derived in vitro or in vivo (Ealy et al., 1993, 1995; Edwards and Hansen, 1997). For example, exposure of lactating dairy cows to high ambient temperatures sufficient to raise rectal temperatures beyond 41.0°C on days 3, 5 or 7 after breeding, did not reduce the proportion of blastocyst-stage embryos recovered 8 d after artificial insemination; whereas, exposure on d 1 did (Ealy et al., 1993). Similarly, in vitro heat stress on day 5 compact morulae did not reduce blastocyst development (Ealy et al., 1995; Edwards and Hansen, 1997).

Because the zygote receives half of its genetic material along with greater than 99% of its cytoplasm from the matured oocyte, the working hypothesis of



the research described herein is that direct effects of heat stress on the maturing oocyte likely perturb developmentally-important processes that carry over to later stage embryos to perhaps increase susceptibility to heat stress. To test this hypothesis, blastocyst development of compact morulae derived from control or heat-stressed oocytes was evaluated after culture at 38.5°C or 41.0°C. Sex ratio of resulting blastocysts was also determined.

The experimental design also allowed for 1) examining direct effects of heat stress on the maturing oocyte to alter ability of resulting embryos to undergo cleavage and compaction after fertilization, and 2) evaluating effects of a physiologically-relevant elevated temperature on compact morulae to possibly alter sex ratio of resulting blastocysts. Previously, Kawarsky and King as reported in Edwards et al. (2001) showed that culture of 8 to 16-cell stage embryos at 42.0°C for 0.5, 2, or 4 h increased the proportion of female embryos that were at the blastocyst stage by 26 to 50% compared to nonheat-stressed controls.

## **Chapter 2**

### **Literature Review**

In order to investigate direct effects of heat stress on the maturing oocyte to possibly carry over to the early embryo to increase its susceptibility to a stressor, it is important to appreciate the challenges of making a blastocyst-stage embryo (Figure 1). This process is inclusive of oocyte maturation, fertilization, embryo cleavage, compaction, and formation of junctional complexes with eventual blastocoele formation. Each of these processes are reviewed in detail in the following sections. In addition, negative effects of heat stress on reproduction, particularly during estrus are described. Furthermore, the direct effects of heat stress on the maturing oocyte and later stage embryos, as well as possible heat-induced alterations in sex ratio are described thereafter.

### **The Making of a Blastocyst**

#### **Oocyte Maturation**

Oocyte maturation is the means by which the oocyte, previously arrested at the dictyate stage of prophase I, resumes meiosis and progresses to metaphase II (**MII**). This process is essential to reduce the number of copies of maternal chromosomes while also enabling the mechanism important for blocking polyspermy (i.e., minimizes the chance of more than one spermatozoon to fertilize the oocyte). This process occurs while the oocyte is resident

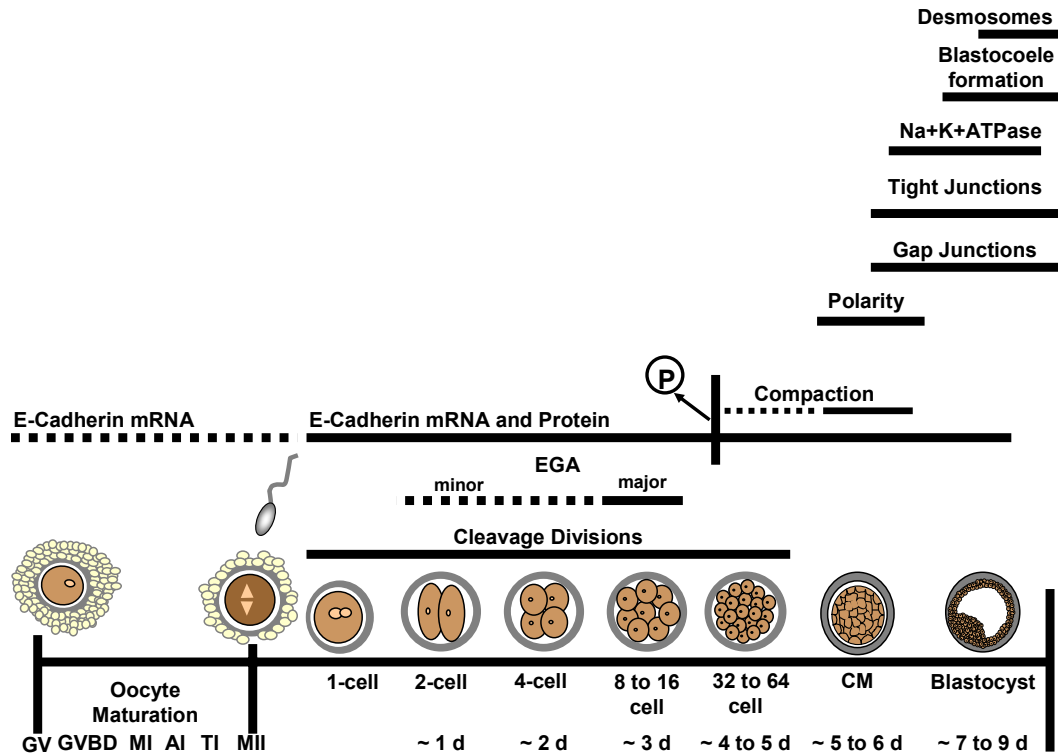


Figure 1. The making of a blastocyst-stage embryo. During oocyte maturation, the germinal vesicle breaks down to allow for progression from the germinal-vesicle (GV) stage of prophase I to metaphase I (MI) where chromosomes align on the metaphase plate, anaphase I (AI) and telophase I (TI), where chromosomes separate and one set of chromosomes is extruded in the form of the first polar body, to then rearrest at metaphase II (MII). At MII, the oocyte is fertilized by a single spermatozoon yielding a 1-cell zygote. Resulting zygotes then undergo a series of mitotic divisions (cleavage) that reduces the volume of cytoplasm of individual blastomeres all the while increasing total cell number. Embryonic genome activation (EGA) must occur by the 8- to 16-cell stage to allow the embryo to synthesize its own transcripts needed for further development. In order for development to proceed, the blastomeres or cells comprising the early embryo must undergo compaction. Although compaction is mediated by E-cadherin at the 16 to 32-cell stage in the bovine (Van Soom et al., 1997) after it has been phosphorylated (Sefton et al., 1992), the mRNA is present within the oocyte and early embryo (Vestweber et al., 1987). Compaction allows for establishment of polarity within the embryo (outer cells become polarized, inner cells remain apolar; Reeve and Ziomek, 1981). In the inner-apolar cells, gap junctions form (Boni et al., 1999) while between the outer-polarized cells tight junctions form (reviewed by Fleming et al., 2000). Compaction, polarity, and formation of junctional complexes are requisite for blastocyst formation. Once the blastocoele forms, a third junctional complex, desmosomes, provide the blastocoele with additional structural support (Fleming et al., 1991).

within the Graafian follicle. Resumption of meiosis is initiated by the luteinizing hormone (**LH**) surge in vivo or occurs spontaneously after removal of the oocyte from its follicular environment (Pincus and Enzmann, 1935). During oocyte maturation, changes occurring in the nucleus are referred to as nuclear maturation, while changes occurring in the cytoplasm are referred to as cytoplasmic maturation.

### ***Nuclear Maturation***

Nuclear maturation is the process by which an oocyte at the germinal-vesicle (**GV**)-stage of prophase I resumes meiosis and progresses to MII. Important to this process is a reduction in chromosome copy number from 4 to 2. Approximate time required to complete this process is approximately 18 to 25 h in bovine oocytes (Kruip et al., 1983; Hyttel et al., 1986a; Sirard et al., 1989; Edwards et al., 2005).

Upon resumption of meiosis, the GV breaks down (**GVBD**) and chromosomes condense (Kubelka et al., 1988). Resumption of meiosis and GVBD requires protein synthesis (Sirard et al., 1989; Lévesque and Sirard, 1996; Lonergan et al., 1998) as use of a protein synthesis inhibitor, cycloheximide, prevented GVBD in bovine oocytes (Tatemoto and Terada, 1995).

Germinal vesicle breakdown and chromosome condensation generally occur within 4 to 12 h after the LH surge (Kruip et al., 1983; Hyttel et al., 1986a) or 4 to 8 h after placement into maturation medium (Hyttel et al., 1986b; Edwards

et al., 2005). By 10 to 12 h, the oocyte has progressed to MI (Kruip et al., 1983; Hyttel et al., 1986a; Edwards et al., 2005) where bivalent chromosomes have aligned on the metaphase plate. Through the contraction of spindle fibers, homologous chromosomes separate (i.e., anaphase I (**AI**) at 12 to 18 h; Kruip et al., 1983; Hyttel et al., 1986a). Thereafter, one set of chromosomes is extruded in the form of the first polar body (i.e., telophase I (**TI**)) at approximately 16 to 21 h of maturation (Kruip et al., 1983; Hyttel et al., 1986a; Edwards et al., 2005). Chromosomes then realign on the metaphase plate and the oocyte rearrests at MII (Kruip et al., 1983; Hyttel et al., 1986a; Edwards et al., 2005) in wait of fertilization.

Resumption of meiosis and progression to MII is in large part mediated by maturation promoting factor (**MPF**) and mitogen-activated protein kinases (**MAPK**). Maturation promoting factor is a multi-unit molecule comprised of a regulatory subunit (i.e., cyclin B; Gautier et al., 1990) and a catalytic subunit (i.e., p34<sup>cdc2</sup> kinase; Labbé et al., 1989). Prior to resumption of meiosis, p34<sup>cdc2</sup> kinase is phosphorylated at threonine 14 and tyrosine 15 (Gould and Nurse, 1989) which renders it inactive. However, after GVBD, dephosphorylation activates MPF (Gautier et al., 1989), presumably by cdc25 phosphatase. Maturation promoting factor levels are low in GV-stage oocytes (Wu et al., 1997; Wehrend and Meinecke, 2001). However, with GVBD, MPF activity increases and peaks at approximately 12 h after resumption of meiosis which is coincident with when the oocyte is at MI. Thereafter, MPF activity decreases and is low by 16 to 20 h during the transition from AI and TI. Maturation promoting activity then

increases to peak again at approximately 20 to 22 h when the oocyte is rearrested at MII (Wu et al., 1997; Wehrend and Meinecke, 2001). Levels of MPF are most likely controlled by de novo synthesis and degradation of cyclin B. Cyclin B levels have been observed to increase before both MI and MII (Wu et al., 1997); whereas, p34<sup>cdc2</sup> levels remain constant through the period of maturation (Lévesque and Sirard, 1996).

However, like MPF, MAPK levels are low in GV-stage oocytes (Fissore et al., 1996) at 8 to 12 h after the resumption of meiosis, MAPK becomes and remains elevated throughout oocyte maturation (Wehrend and Meinecke, 2001). It has been hypothesized that the lack of decline in MAPK levels during the transition from MI to MII may be important for preventing the oocyte from entering interphase, thereby preventing DNA replication (reviewed by Fan and Sun, 2004).

### ***Cytoplasmic Maturation***

Cytoplasmic maturation induces changes in the cytoplasm important for preventing polyspermy (Hosoe and Shioya, 1997) and conferring developmental competence of the oocyte after fertilization (reviewed by Sirard et al., 2006). This process occurs concomitant with nuclear maturation and in total takes approximately 24 to 30 h to complete (Hyttel et al., 1986b; Edwards et al., 2005).

During cytoplasmic maturation, the oocyte undergoes structural changes within its cytoskeleton. Microtubules transition from being disorganized at the GV-stage, to forming asters near the condensed chromatin during GVBD, to

being localized around the meiotic spindle during MI and MII (Kim et al., 2000). Additionally, microfilament organization changes also occur. Following GVBD, microfilaments transition from being organized in close association with the GV in the cortex of the oocyte, to being associated with maternal chromatin. At MII, microfilaments are organized around both the maternal chromatin and the polar body (Kim et al., 2000).

Another prominent change occurring within the oocyte during cytoplasmic maturation is the redistribution of organelles, particularly that of mitochondria, smooth endoplasmic reticulum (**SER**), rough endoplasmic reticulum (**RER**), and ribosomes. When the oocyte is at the GV-stage, mitochondria are located peripherally (Kruip et al., 1983; Hyttel et al., 1986a). As the oocyte undergoes GVBD and progresses to MI, mitochondria show clustering and close association with lipid droplets and SER (Kruip et al., 1983; Hyttel et al., 1986a). Coincident with MII, mitochondria assume an even distribution within the ooplasm (Kruip et al., 1983; Hyttel et al., 1986a). While SER in GV-stage oocyte show well developed cisternae that are often associated with mitochondria; by MII-stage, SER decreases in size and experience reduced contact with mitochondria (Hyttel et al., 1986a). Unlike SER, RER is present in GV-stage oocytes but in large part are not detectable after resumption of meiosis (Kruip et al., 1983). In contrast, ribosomes are largely absent in GV-stage oocytes. Ribosomes do not become detectable until the late MI stage and increase in abundance at MII (Kruip et al., 1983).

Translocation of cortical granules (**CG**) to the oolemma is a hallmark of cytoplasmic maturation. At the GV-stage, CG appear as large aggregates which are located predominantly within the periphery of the ooplasm associated with the SER and golgi complex (Kruip et al., 1983; Hyttel et al., 1986a; Hosoe and Shioya, 1997). After resumption of meiosis, CG begin to disperse and migrate towards the oolemma appearing as small aggregates with some dispersion (Kruip et al., 1983). When oocyte maturation is complete, CG are completely translocated and dispersed to the oolemma (Kruip et al., 1983; Hyttel et al., 1986a). Translocation of CG to the oolemma is a critical process for preventing polyspermy. For example, an increase in polyspermic penetration from 6.0% to 59.6% was observed in oocytes with a higher distribution of CG aggregates 5 h after fertilization compared to those oocytes with a higher proportion of dispersed CG (Hosoe and Shioya, 1997).

During oocyte maturation, changes in protein synthesis, transcription, and abundance of polyadenylated mRNA (**Poly(A)**) mRNA also occur. After resumption of meiosis, protein synthesis increases for the first 8 to 12 h but decreases thereafter (Kastrop et al., 1990; Tomek et al., 2002; Coenen et al., 2004). Tomek et al. (2002) measured [<sup>35</sup>S]methionine incorporation in pools of 50 oocytes and showed that protein synthesis increased three-fold up to 10 h of in vitro maturation (**IVM**) compared to those oocytes at prophase I. However, protein synthesis decreased thereafter in MI and MII stage oocytes assessed at 14 to 18 and 20 to 24 h, respectively (Tomek et al., 2002). In agreement with this, Coenen et al. (2004) demonstrated a decrease in the number of proteins



synthesized in oocytes at MI and MII compared to those oocytes at the GV-stage (392, 345, and 520 proteins synthesized in MI, MII, and GV-stage oocytes, respectively) when evaluated after a 4 h of culture in [<sup>35</sup>S]methionine.

Transcriptional activity of the oocyte has also been shown to decrease over the span of maturation. In MII oocytes, <sup>35</sup>S-UTP uptake was lower compared to those at the GV-stage (Memili et al., 1998). Although total RNA generally remains unchanged during oocyte maturation (Lequarre et al., 2004), the abundance of Poly(A) mRNA decreases markedly. Payton and Edwards (2005) showed that Poly(A) mRNA decreased from 78.2 to 27.4 pg/oocyte after 24 h of in vitro maturation.

## **Fertilization**

Fertilization may be defined as the fusion of a spermatozoon with a MII oocyte. Fertilization is important for initiating oocyte activation (i.e., resumption and completion of meiosis) and results in the formation of a 1-cell zygote. In order for a spermatozoon to fertilize an oocyte, it first must undergo capacitation. Capacitation is the process through which alterations in the plasma membrane enable the spermatozoon to bind to the zona pellucida (**ZP**) and undergo the acrosome reaction (reviewed by Suarez, 2007). Capacitation is associated with hypermotility of the spermatozoon and has been hypothesized to assist in the detachment of the spermatozoon from the oviductal epithelium (Lefebvre and Suarez, 1996).

Currently, data on the presence or absence of cumulus cells at the time of fertilization in the bovine are conflicting. Earlier studies observed a lack of cumulus (Lorton and First, 1979), while later studies reported its presence during and after fertilization (Hyttel et al., 1988). Nonetheless, in order for the spermatozoon to fertilize the oocyte, it must first bind and pass through the ZP, an outer “shell” constructed from glycoproteins which is secreted by the oocyte during its growth phase (reviewed by Wassarman et al., 1996). Thus far, at least 4 mammalian ZP glycoproteins have been isolated (reviewed by Sinowatz et al., 2001). Binding of a spermatozoon to the ZP3 receptor induces the acrosome reaction (Hinsch et al., 2005). During the acrosome reaction, fusion of the spermatozoon nuclear membrane to the inner acrosomal membrane results in the release of hydrolytic enzymes such as acrosin and hyaluronidase that digests the ZP (reviewed by Saling, 1991). Once through the ZP, the spermatozoon may then bind and fuse with the oolemma. Binding of the spermatozoon to the oolemma may be mediated by interactions between ADAMS (a disintegrin and a metalloprotease domain) on the spermatozoon and integrins on the oocyte (Campbell et al., 2000; reviewed by Evans, 2001). Binding of the spermatozoon to the oolemma may initiate intracellular  $\text{Ca}^{2+}$  oscillations most likely through activation of the phosphoinositide pathway (Fissore et al., 1995; reviewed by Malcuit et al., 2006).

After fusion, spermatozoon-mediated increases in  $\text{Ca}^{2+}$  oscillations result in the release of CG contents into the preivitelline space as a means to prevent polyspermy (Gardner and Evans, 2006). Additionally,  $\text{Ca}^{2+}$  oscillations are also

important for oocyte activation (i.e., re-initiation and completion of meiosis). Oocyte activation is initiated through cyclin B degradation (Nixon et al., 2002; Marangos and Carroll, 2004) and MPF inactivation. Inactivation of MPF allows for resumption of meiosis allowing the oocyte to progress through AII and TII and extrude its second polar body. Completion of meiosis results in a haploid oocyte having only 1 copy (set) of chromosomes.

Upon fusion with the MII oocyte, the spermatozoon's nuclear envelope breaks down, chromosomes decondense, and protamines are replaced with histones (McLay and Clarke, 1997). Following this, reformation of the nuclear envelope occurs (Laurincik et al., 1998) as a first step towards pronuclear formation. Maternal and paternal pronuclei (**PN**) formation occurs approximately 7 to 8 h after ovulation in vivo or 5 to 7 hpi in vitro (in vivo- Hyttel et al., 1988; in vitro- Laurincik et al., 1998). Pronuclear formation is coincident with the completion of meiosis within the oocyte (McLay and Clarke, 1997; reviewed by McLay and Clarke, 2003). After PN formation, both migrate towards one another and become apposed in a process called syngamy.

## **Cleavage of the Early Embryo**

Because the zygote receives more than 99% of its cytoplasm from the oocyte, it is much larger than a somatic cell (110 to 150  $\mu\text{m}$  in size compared to 10 to 20  $\mu\text{m}$ ). Therefore, the zygote must undergo cleavage divisions whereby a single blastomere divides yielding smaller blastomeres in order to increase cell number while reducing individual cell size. The first cleavage division can be

initiated as early as 24 to 26 h after IVF (Laurincík et al., 1998; Ward et al., 2001).

In order to decrease cytoplasmic volume, the embryo undergoes an altered cell cycle which mainly consists of the DNA-synthesis (S-phase) and mitosis (M-phase). In the second and third cell cycle (cleavage to the 4 and 8-cell embryo), gap phase 1 (**G1**) and gap phase 2 (**G2**; i.e., phases of cell cycle in which replication of cytoplasmic organelles occurs) are abbreviated or absent resulting in a shorter cell cycle lasting approximately 12 to 13 h each (Barnes and Eyestone, 1990). The fourth cell cycle (16-cell embryo) is marked by the return of both G1 and G2 (Barnes and Eyestone, 1990) resulting in a longer cell cycle lasting anywhere from 35 to 48 h (Holm et al., 1998; Lequarre et al., 2003).

Timing of the first cleavage relative to fertilization has been correlated with developmental competence of the embryo. Specifically, Lonergan et al. (1999) demonstrated that those zygotes that cleaved by 30 hpi yielded higher blastocyst development and hatched from the ZP than their counterparts that cleaved at 36 or 42 hpi (61.1, 34.7, and 5.9% development to blastocyst and 96, 35, and 0% hatched in those that cleaved at 30, 36, or 42 hpi, respectively). In addition, timing of the first cleavage and percentage of zygotes that undergo cleavage has also been shown to be influenced by the fertility status of the sire and quality of semen (Ward et al., 2001). Additionally, Comizzoli et al. (2000) demonstrated that use of higher quality bull ejaculates hastened the onset of the first S-phase by 2 h and improved development to the blastocyst stage (38.4% and 8.2% in high quality and low quality ejaculates, respectively).

## Embryonic Genome Activation

After resumption of meiosis, the oocyte becomes largely transcriptionally inactive and therefore must rely on maternal pools of RNA previously accumulated during the growth phase of oogenesis (reviewed by Rodriguez and Farin, 2004). Transcriptional quiescence continues during fertilization and during the first few cleavage divisions of the early embryo. During this time the embryo transitions from being reliant on maternal transcripts to being dependant on its own transcripts to support further embryonic development (reviewed by Telford et al., 1990). The onset of embryonic genome activation (**EGA**) is species dependent with EGA occurring at the late 1-cell in mouse (Aoki et al., 1997), 4 to 8-cell in the rabbit (Van Blerkom and Manes, 1974) and humans (Braude et al., 1988), and 8 to 16-cell stage in cattle (Camous et al., 1986) and sheep (Crosby et al., 1988).

In the bovine, a decline in protein and RNA abundance is evident beginning during oocyte maturation and continues to the 8 to 16-cell stage. Thereafter, protein and RNA abundance increases thereafter which is coincident with the onset of EGA. Frei et al. (1989) demonstrated that [<sup>35</sup>S]methionine incorporation decreased approximately 3-fold from the post ovulatory oocyte to the 8-cell stage embryo; however, there was a 20-fold increase from the 16-cell stage embryo to the blastocyst-stage embryo (Frei et al., 1989). Frei et al. (1989) was also unable to detect RNA synthesis until the 16-cell stage in bovine embryos. Unlike with RNA synthesis, Vigneault et al. (2004) used qPCR (Real-Time PCR) to demonstrate that mRNA of several transcripts decline in

abundance from the GV to 8-cell stage but then increases thereafter which is coincident with EGA. Even though major transcriptional activity associated with EGA has been shown to occur at the 8 to 16-cell stage, minor transcriptional activity has been observed in early stage embryos. In fact, Memili and First (1999) used inhibitors aphidicolin (DNA synthesis inhibitor),  $\alpha$ -amanitin (transcription inhibitor) and Trichostatin A (histone deacetylase inhibitor) to detect minor transcription prior to EGA as early as the 1-cell stage.

### **Compaction and the Role of E-Cadherin**

After the initial cleavage divisions, the morula-stage embryo must undergo compaction before forming a blastocyst. Compaction is the process by which the blastomeres or cells comprising the preattachment embryo flatten against one another (Ducibella and Anderson, 1975). This flattening of cells results in increased cell to cell contact between adjacent blastomeres within the embryo (Pratt et al., 1982). Compaction occurs at the 8-cell stage in mice (Ducibella and Anderson, 1975), 16 to 32-cell stage in the bovine (Van Soom et al., 1997), and 32 to 64-cell stage in the rabbit (Koyama et al., 1994). Furthermore, compaction is reported to be mediated by E-cadherin (uvomorulin; reviewed by Kemler, 1993),  $\text{Ca}^{2+}$  (Ducibella and Anderson, 1975), and protein kinase C (**PKC**; Winkel et al., 1990).

E-cadherin is a protein from the cadherin family that is important for cell adhesion during cleavage of the early embryo. In murine embryos, culture with

antibodies against E-cadherin protein prevented compaction and subsequent blastocyst formation (Watson et al., 1990).

Although compaction does not occur until the 16- to 32-cell stage in bovine embryos, the mRNA E-cadherin is present in the maturing oocyte through the compact morula-stage embryo (Barcroft et al., 1998; Nganvongpanit et al., 2006). During early cleavage divisions, E-cadherin adheres blastomeres together through homophillic binding between two E-cadherin molecules. This homophillic binding is mediated by the extracellular domain of E-cadherin which contains five tandem portions linked together by  $\text{Ca}^{2+}$  binding sites called cadherin repeats (reviewed by Aberle et al., 1996). Additionally,  $\text{Ca}^{2+}$  is important for compaction. Culture of 4 to 8-cell murine embryos in  $\text{Ca}^{2+}$  free media resulting in the inhibition of compaction altogether (Ducibella and Anderson, 1975). Moreover, blocking both intracellular and extracellular  $\text{Ca}^{2+}$  through the use of EGTA and a  $\text{Ca}^{2+}$  channel blocker, respectively, resulted in decompaction of 8-cell murine compact morulae (Pey et al., 1998).

In order for compaction to occur, phosphorylation of E-cadherin is important (Sefton et al., 1992). Phosphorylation results in the migration of E-cadherin from an apolar distribution throughout the cytoplasm to being localized to sites where blastomeres are in direct contact with one another (Vestweber et al., 1987). Although the exact mechanism through which E-cadherin becomes phosphorylated is unclear, PKC has been implicated. For example, activation of PKC by phorbol esters or diacylglyceride induces premature compaction in 4-cell murine embryos (Winkel et al., 1990).

After phosphorylation of E-cadherin, adhesion of blastomeres within the compacting morula is enhanced through the assembly of a complex referred to as an adherens junction. Each junction requires the interaction of multiple domains of adjacent blastomeres (reviewed by Kemler, 1993). Specifically, the intracellular part is comprised of multiple cytosolic proteins,  $\alpha$ -catenin and  $\beta$ -catenin, that link E-cadherin to the cytoskeleton (Ozawa et al., 1989).

In the mouse, the assembly of the adherens junction begins with the interaction of E-cadherin with the cytoplasmic domain of  $\beta$ -catenin (Ozawa and Kemler, 1992). Beta-catenin is a cytoplasmic plaque protein that has both maternal and embryonic origins (Ohsugi et al., 1999). Similar to E-cadherin,  $\beta$ -catenin is also phosphorylated to promote adherens junction assembly (Pauken and Capco, 1999; Goval et al., 2000). The importance of  $\beta$ -catenin in compaction has been demonstrated by a decrease in cell adhesion observed with truncation of the  $\beta$ -catenin allele (De Vries et al., 2004). After  $\beta$ -catenin associates with E-cadherin, this complex then indirectly is linked to the cytoskeleton by another cytoplasmic plaque protein,  $\alpha$ -catenin. Alpha-catenin binds to  $\beta$ -catenin and then to actin, therefore completing the link between two adjacent blastomeres resulting in compaction of adjacent blastomeres (Aberle et al., 1994).

### ***Blastomere Polarization within the Preattachment Embryo***

Compaction of the cleavage-stage embryo is an important milestone in embryonic development as it facilitates the differentiation of the first epithelium



(reviewed by Yamanaka et al., 2006). The physical act of compaction allows for the formation of two distinct populations of cells in the embryo (Johnson and Ziomek, 1981). The outer blastomeres of the embryo become polarized and have microvilli covering the apical border, while the inner blastomeres lack this microvillus cap and remain apolar (Reeve and Ziomek, 1981). Polar cells located in the outermost layer of the embryo give rise to the trophectoderm, a layer of epithelial cells that will differentiate to give rise to the placenta. The inner, apolar cells differentiate into the inner cell mass (**ICM**) which will make up the embryo proper (reviewed by Yamanaka et al., 2006).

Whether a blastomere within the compact morula will differentiate into a polar or apolar cell is regulated by cell to cell contact and the relative position of the blastomere within the compact morulae (Hillman et al., 1972). For example, when disaggregated 8-cell embryos are cultured alone they do not polarize; however, when those cells are re-aggregated into couplets, polarity can be re-established at points opposite that cell to cell contact (Ziomek and Johnson, 1980).

### ***Formation of Gap Junctions in the Preattachment Embryo***

Gap junctions are intercellular connections that form between apolar cells (i.e., inner blastomeres of the compact morula that lack apical microvilli) after compaction and permit cell to cell communication within the early embryo (reviewed by Bruzzone et al., 1996). Gap junctions allow for the passage of small molecular weight ions (<1000 daltons in size; reviewed by Bruzzone et al.,

1996) such as chloride (**CL<sup>-</sup>**), sodium (**Na<sup>+</sup>**), potassium (**K<sup>+</sup>**), cyclic adenosine monophosphate (**cAMP**), and Ca<sup>2+</sup> as well as amino acids and nucleotides but not nucleic acids, proteins or polysaccharides (Krysko et al., 2005; Ponsioen et al., 2007).

Each gap junction is composed of six transmembrane proteins called connexins which aggregate together to form a connexon (Unger et al., 1999). The localization of connexons is not isolated to the preattachment embryo, rather multiple forms of connexins are expressed in rat ovaries, placenta and surrounding epithelium (Risek et al., 1990; Pauken and Lo, 1995). While two types of connexon proteins are predominately expressed in the murine embryo; (Connexin 43 (**Cx43**) and Connexin 32), Cx43 seems to be the predominant form involved in regulation of embryonic development in both the murine and bovine embryo (De Sousa et al., 1993; Wrenzycki et al., 1996).

Gap junction assembly is critical for the maintenance of compaction. Bevilacqua et al. (1989) demonstrated that injection of antisense gap junction mRNA into 2- and 4-cell murine embryos resulted in only 28 and 20% of injected embryos undergoing compaction at the 8-cell stage. Additionally, injection of gap-junction antibodies into 2-cell murine embryos resulted in abnormal compaction with only half of the blastomeres within a given embryo undergoing compaction at the 8-cell stage (Lee et al., 1987). This same study also demonstrated a decrease in the number of 8-cell embryos showing Lucifer Yellow dye transfer in response to injection of gap-junction antibodies (86 and

29% in embryos not injected and injected embryos, respectively; Lee et al., 1987).

In the mouse, gap junctions are first apparent at the time of compaction at the 8-cell stage (Goodall and Johnson, 1984). However, the mRNA and protein for Cx43 can be detected as early as the 4-cell stage (De Sousa et al., 1993). In the bovine, Cx43 mRNA is detectable in maturing oocyte through the blastocyst stage embryo (Wrenzycki et al., 1996; Lonergan et al., 2003). However, functional gap junctions do not become detectable by electron microscopy until the morula or blastocyst stage (Boni et al., 1999). In morula or blastocyst-stage embryos, functional gap junctions have been detected in the cells within the ICM and as well as those between the ICM and trophectoderm. However, gap junctions are absent from the trophectoderm (Boni et al., 1999).

Like other junctional complex proteins, Cx43 undergoes post-translational modifications to initiate assembly into the membrane (Warn-Cramer et al., 1996; Lampe et al., 2000; Lampe and Lau, 2004). Phosphorylation of Cx43 has been shown to take place in the golgi or endoplasmic reticulum in rat mammary tumor cells (Laird et al., 1995) and may be linked to PKC (Lampe et al., 2000). Phosphorylation of Cx43 regulates gap junction communication by mediating Cx43 protein migration to the plasma membrane, gap junction assembly, and turnover (Lampe and Lau, 2004).

## ***Formation of Tight Junctions in the Preattachment Embryo***

Compaction is a prerequisite of tight junction assembly (Watson and Barcroft, 2001). In particular, tight junctions form belt-like complexes in the apical domain of the outer-polarized cells (i.e., cells with apical microvilli that differentiate into trophectoderm cells; Fleming et al., 2000; Violette et al., 2006). Tight junctions are important for restricting the movement of fluids within the developing embryo in order to regulate blastocyst formation (Fleming et al., 2001). Tight junctional complexes are comprised of several proteins, namely zonula occludens (**ZO**; Fleming 1989; Sheth et al., 1997), rab 13 (Sheth et al., 2000a), cingulin (Fleming et al., 1993), and occludin (Furuse et al., 1994).

Tight junction assembly is initiated after compaction of the 8-cell stage murine embryo and occurs in a stepwise process (Fleming et al., 1989). First, ZO-1 $\alpha$ - and rab 13 co-localize at the blastomere membrane (Fleming et al., 1989; reviewed by Fleming et al., 2000; Sheth et al., 2000a). ZO-1  $\alpha$ - is an isoform of ZO-1 that is a result of alternative RNA splicing (Willott et al., 1992) and lacks the 80 amino acid sequence in the C-terminus (Balda and Anderson, 1993). Furthermore, both ZO-1 $\alpha$ - and rab 13 mRNA is detectable from the maturing oocyte to blastocyst even though assembly does not commence until after compaction (Sheth et al., 1997, 2000).

Approximately 12 h after co-localization of ZO-1 $\alpha$ - and rab 13, cingulin is transported to the cell surface at points of blastomeric contacts (Fleming et al., 1993). Cingulin connects the cytoskeleton to the previously assembled complex as evident by studies showing that a part of it interacts with ZO-1, ZO-2, and ZO-

3 proteins (Cordenonsi et al., 1999). The last and possibly most important protein to assemble a tight junction is ZO-1 $\alpha$  and occludin. Unlike the other tight junction proteins, ZO-1  $\alpha$  is synthesized de novo at the morula stage and can be observed in the progenitor cells of the trophectoderm and only faintly in those of the ICM (Fleming and Hay, 1991). Additionally, ZO-1  $\alpha$  co-localizes with occludin and assembles at the blastomeric membranes (Furuse et al., 1994; Eckert et al., 2005). In the absence of co-localization between occludin and ZO-1  $\alpha$ , occludin does not assemble at the tight junction (Sheth et al., 2000b).

Currently, the literature has limited data on tight junctions in preattachment bovine embryos. Miller et al. (2003) used semiquantification of reverse transcription-PCR to determine that tight junctional mRNA (ZO-1, ZO-1 $\alpha$ , occludin, and ZO-2) is present from the GV-stage oocyte to the blastocyst stage. However, the proportion of embryos expressing those transcripts declines from the oocyte to the 8-cell stage (i.e., prior to EGA) but then increases at the morula stage. Unlike the remaining tight junction proteins, ZO-1  $\alpha$  protein is likely of embryonic origin as it is detectable at the morula stage (Barcroft et al., 1998).

## **Formation of the Blastocyst-Stage Embryo**

After formation of tight junctions in the embryo following compaction, a blastocoele (i.e., fluid filled cavity) is generated through fluid movement, mainly water, across the outer primitive epithelium (reviewed by Biggers et al., 1988). This influx of water into the blastocoele cavity requires interaction between several junctional complexes previously assembled in the preattachment embryo

as well as  $\text{Na}^+\text{K}^+\text{ATPase}$  activity and aquaporins (reviewed by Watson and Barcroft, 2001).

### ***$\text{Na}^+\text{K}^+\text{ATPase}$ Activity during Blastocoele Formation***

$\text{Na}^+\text{K}^+\text{ATPase}$  is a multi-protein complex consisting of an  $\alpha$ - and  $\beta$ -subunit. The  $\alpha$ -subunit is thought to be the active or catalytic subunit (Jorgensen, 1982). In contrast, the  $\beta$ -subunit is glycosylated and is responsible for the incorporation of the  $\alpha$ -subunit into the plasma membrane (reviewed by Kaplan, 2002). Moreover, an additional subunit (i.e., the  $\gamma$ -subunit) may also play a role in blastocyst formation as it displays a similar distribution and expression pattern as both  $\alpha$  and  $\beta$ -subunits (Barcroft et al., 2002). Establishment of an osmotic gradient enabling the transport of water across developing epithelium is facilitated by  $\text{Na}^+\text{K}^+\text{ATPase}$  (reviewed by Watson et al., 2004).

$\text{Na}^+\text{K}^+\text{ATPase}$  activity within the compacted embryo is critical for blastocoele formation and has been studied using ouabain, a  $\text{Na}^+\text{K}^+\text{ATPase}$  inhibitor (Betts et al., 1997). Culture of blastocyst-stage embryos in medium containing cytochalasin-D (induces blastocoele collapse), followed by ouabain for 12 h, inhibited bovine embryos from re-expanding (Betts et al., 1997).

Bovine embryos express  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$  along with  $\beta 1$  and  $\beta 2$   $\text{Na}^+\text{K}^+\text{ATPase}$  subunits (Betts et al., 1997; Watson and Barcroft, 2001). Even though  $\text{Na}^+\text{K}^+\text{ATPase}$  does not become a functional unit until the late compact morula stage, the mRNA responsible for encoding  $\alpha$ - and  $\beta$ -gene isoforms are detectable by reverse-transcription PCR from the 1-cell zygote to the blastocyst-

stage embryo (Betts et al., 1997). Interestingly, De Sousa et al. (1998) detected the  $\alpha 1$  mRNA within the maturing oocyte. Additionally,  $\text{Na}^+\text{K}^+\text{ATPase}$  subunits maintain an apolar distribution prior to compaction. However, at the 16 to 32-cell stage, it has been shown that the  $\alpha 1$ -subunit becomes restricted to the basolateral portion of the trophectoderm while the  $\beta 1$ -subunit is localized to the apical domain (Betts et al., 1998). In addition to being present in the trophectoderm, the  $\alpha 1$  subunit mRNA has also been detected within the ICM (Wrenzycki et al., 2003).

While all  $\alpha$ -subunits and  $\beta 2$ -subunit are expressed prior to compaction,  $\beta 1$  is transcribed at the morula stage (Betts et al., 1997). This late emergence of  $\beta 1$  suggests that it may play a role in the activation of  $\text{Na}^+\text{K}^+\text{ATPase}$  to facilitate blastocoele formation. Once assembled, the  $\text{Na}^+\text{K}^+\text{ATPase}$  pump works similar to those found in other systems such as the kidneys by transferring 3  $\text{Na}^+$  ions out of the cell and transporting 2  $\text{K}^+$  ions to enter the cell through a series of conformational changes brought about by ATP hydrolysis (Feraille and Doucet, 2001).

### ***Aquaporins in Blastocoele Formation***

Aquaporins are channels that allow the passage of water into a cell and are important for blastocoele formation (reviewed by Huang et al., 2006). It has been shown that murine preimplantation embryos express several aquaporin (**AQP**) protein isoforms. Specifically, murine embryos express AQP1, 3, 5, 7, 8, and 9 (Offenberg et al., 2000). Moreover, localization of AQP in both the apical

and basolateral domains of the trophectoderm (Barcroft et al., 2003) suggests that the polarization of AQP isoforms may contribute to the transcellular movement of water across the plasma membrane of trophectoderm cells. Furthermore, aquaporin activity can be altered by molecules such as sucrose that disrupt the osmotic gradient generated by  $\text{Na}^+\text{K}^+\text{ATPase}$  (Barcroft et al., 2003).

### ***Desmosomes***

As the blastocoele cavity begins to form, desmosomes form within the embryo. Desmosomes are junctional complexes that become localized to the trophectoderm and are closely associated with tight junctions (Watson et al., 1990). The main function of desmosomes is to facilitate cellular integrity by attaching to intermediate filaments of the embryo's cytoskeleton (Jackson et al., 1980). Each desmosome is comprised of three cytoplasmic plaque proteins (desmoplakins, plakoglobin, and plakophilins) and two cadherins proteins (desmocollins and desmogleins (desmocollins **(Dsc)** and desmogleins **(Dsg)**; North et al., 1999). Moreover, multiple isoforms have been isolated for each protein (Fleming et al., 1991).

Desmosomes ensure structural integrity and cell adhesion through heterophilic binding between Dsc and Dsg in adjacent epithelial cells (Chitaeu and Troyanovsky, 1997). Desmosomes are first noted at the 16 to 32-cell in murine embryos and undergo a marked increase in expanding blastocysts (Fleming et al., 1991). Similar to the murine embryo, bovine desmosomes are



present in the blastocyst stage embryo and can still be noted at day 12 of development between epithelial cells (Vejlsted et al., 2006).

## **Heat Stress and its Impact on Reproduction in the Dairy Cow**

Female dairy cows experience a decline in milk yield (reviewed by West, 2003) and reproductive performance when exposed to high environmental temperatures (reviewed by Hansen, 2007). Heat stress is a world-wide phenomenon that reduces fertility by decreasing pregnancy rates in lactating dairy cattle (reviewed by Hansen and Aréchiga, 1999; De Renis and Scaramuzzi, 2003; and Hansen, 2007).

Reductions in pregnancy rates in heat-stressed dairy cattle may be attributable to inaccurate estrus detection due to shortened expression or intensity of estrus (Gangwar et al., 1965). A decrease in the duration of estrus from 14.0 h in cool months (January to April) to 8.2 h in the warm months (July to September) has been documented (Monty and Wolff, 1974). Additionally, while the frequency of estrus-mounting activity has been shown to decrease with hot weather, the duration between subsequent mountings increases (Pennington et al., 1985). Taken together, for a dairy operation that relies on visual heat detection, these factors result in an increased number of services (times a cow is bred) per conception and an increased number of days that a cow will remain open (i.e., not pregnant; Poston et al., 1962).

Heat stress may also alter follicular and corpus luteum function. Reduction in size of the first wave dominant follicle has been noted in heat-stressed dairy

cows (Badinga et al., 1993). Reports of estradiol concentrations have been conflictive as studies have reported no change (Badinga et al., 1993; Guzeloglu et al., 2001), a decrease (Wolfenson et al., 1995), or an increase (Rosenberg et al., 1982) in heat stress conditions. Nonetheless, findings by Wolfenson et al. (1995) showed that ovulation is not altered in heat-stressed cows compared to those that were exposed to shade and sprinkler systems. After ovulation, the growth and maximum size of the resulting corpus luteum remains unaltered by chronic stress (Howell et al., 1994). Despite this, both an increase (Roman-Ponce et al., 1981) and a decrease (Howell et al., 1994; Wolfenson et al., 2002) in progesterone (**P<sub>4</sub>**) during the luteal phase of the estrous cycle have been noted. These reports are conflictive with those reporting no change in P<sub>4</sub> concentrations during heat stress (Monty and Racowsky, 1987; Badinga et al., 1993).

While many factors likely contribute to reduced fertility in heat-stressed dairy cows, elevations in body temperature in response to elevated ambient temperatures may be most detrimental. Morton et al. (2007) showed as maximum temperature-humidity index increased from approximately 66 to 78, conception rates decreased from 47 to 26%. Additionally, Cavestany et al. (1985) found that conception rates declined from approximately 32.5 to 2.5% with an increase in maximum air temperature from 20 to 35°C, respectively. High ambient temperatures, such as those experienced by dairy cattle during the summer, are responsible for elevations in rectal temperatures (Sartori et al., 2002). Data have shown that rectal temperatures can reach or exceed 41.0°C in

heat-stressed dairy cattle (Monty and Wolff, 1974; Ealy et al., 1993). Furthermore, rectal temperatures are highly correlated to uterine temperatures (Gwazdauskas et al., 1973). There is a strong negative correlation of elevated rectal temperatures with reduced fertility as a reduction in pregnancy rate has been observed with each 1°C increase in rectal temperature (Ulberg and Burfening, 1967).

### **Effects of Heat Stress during Estrus to Decrease Fertility**

While exposure to heat stress is not an isolated event and therefore may exert negative effects on multiple embryonic stages (Ealy et al., 1993, 1995), it is most problematic during estrus (i.e., when the maturing oocyte is resident within the Graafian follicle). Specifically, elevations in ambient temperatures prior to breeding results in a reduction in conception rates (Gwazdauskas et al., 1973). For example, Holstein cows exposed to ambient air temperatures of 107°F before breeding experienced a conception rate of 31.0% compared to 61.5% in those exposed to 92°F before breeding (Stott and Williams, 1962). During heat stress reductions in conception rates are coincident with heat-induced elevations in both uterine and rectal temperatures (Gwazdauskas et al., 1973). Fallon (1962) observed that elevations in rectal temperatures from 101.7 to 105.5°F on the day of breeding were coincident with reduced fertility. Reductions in fertility have also been reported in Zebu cattle when heat stress was great enough to elevate rectal temperature (Zakari et al., 1981). Specifically, pregnancy rates declined from 52 to 12% as rectal temperatures increased 1 to 2°C (38.2 to 39.1

versus 39.2 to 40.0°C). While an attempt to alleviate the negative effects of heat stress on fertility by cooling heat-stressed cows for 24 h prior to breeding resulted in a partial increase in conception rates from 17 to 38%, the negative consequences of heat stress to decrease fertility were not fully eliminated (Stott and Wiersma, 1976).

Reductions in fertility observed in heat-stressed cows are likely due to heat-induced elevations in body temperature to negatively impact the maturing oocyte. Putney et al. (1989) exposed superovulated-Holstein heifers to an elevated ambient temperature of 42.0°C in environmentally-controlled chambers for the first 10 h of estrus. Doing so elevated rectal temperatures to 41.3°C. After removal from the chambers, heifers were placed in evaporatively-cooled barns to reduce rectal temperatures to 38.8°C prior to artificial insemination. Seventy-six percent of those embryos recovered from heat-stressed cows on day 7 were assessed as poor to fair quality with only 4% classified as good to excellent quality. In contrast, 42.1 and 47.4% of embryos recovered from nonheat-stressed cows were assessed as fair to poor and good to excellent. Moreover, 68% of heat stress-derived embryos were deemed abnormal compared to only 21.1 in nonheat-stressed embryos (i.e., not of expected stage with or without the presence of extruded or degenerate blastomeres; Putney et al., 1989).

Heat stress applied prior to mating is not without consequence in other species. In a study conducted by Baumgartner and Chrisman (1987), mice were heat-stressed at  $35.0 \pm 1^\circ\text{C}$  and  $65 \pm 3\%$  relative humidity for 12.5 h following

injection with human chorionic gonadotropin (**hCG**) sufficient to raise rectal temperatures 1 to 2°C. Ovulated oocytes recovered 13.5 h after hCG showed inhibition of nuclear maturation as 54.3% versus 91.6% had progressed to MII in heat-stressed versus control oocytes, respectively. Similarly, Baumgartner and Chrisman (1981) observed an increase in proportion of murine oocytes arrested at the MI stage (12.3 versus 0% in control and heat-stressed oocytes, respectively) when recovered after hCG injection from mice exposed to  $35.0 \pm 1^\circ\text{C}$  and  $65 \pm 3\%$  relative humidity for 15.5 h. Other abnormalities such as dispersed or degenerating chromosomes configurations or fragmented nuclei in oocytes obtained from heat-stressed mice were also reported (Baumgartner and Chrisman, 1981, 1987).

### ***In Vitro Model to Study Direct Effects of Heat Stress on the Maturing Oocyte***

An end consequence of elevated body temperatures in heat-stressed dairy cows is to compromise the maturing oocyte thereby reducing embryonic development after fertilization. Ability to mature bovine oocytes in vitro allows for removal of the oocyte from the maternal environment and assessing direct effects of heat stress. Use of in vitro maturation is an appropriate model to assess the direct effects of elevated body temperature on the maturing oocyte as both in vivo and in vitro maturation parallel. Also, reductions in embryonic development of heat-stressed oocytes are similar regardless of oocyte maturation origin (in vivo or in vitro).

It has been demonstrated that for both in vitro and in vivo derived oocytes, GVBD occurs between 4 to 8 h after the resumption of meiosis (in vitro-Hyttel et al., 1986b; Sirard et al., 1989; Edwards et al., 2005; in vivo-Kruip et al., 1983; Hyttel et al., 1986a). Additionally, in vivo and in vitro derived oocytes progress to MI and MII by 10 to 12 and 18 to 25 h, respectively and extrude a polar body by 16 to 21 h (in vitro- Hyttel et al., 1986b; Sirard et al., 1989; Edwards et al., 2005; in vivo-Kruip et al., 1983; Hyttel et al., 1986a). Moreover, cytoplasmic maturation of in vivo derived oocytes is comparable to in vitro matured oocytes with respect to the migration of organelles such as SER, RER, ribosomes, and cortical granules (Kruip et al., 1983; Hyttel et al., 1986 a,b).

Furthermore, in vitro derived embryos result in similar pregnancy and calving rates after embryo transfer (Hasler et al., 2000) compared to in vivo derived embryos. In vivo derived embryos also show similar reductions in development in response to heat stress. In fact, maturing oocytes at 41.0°C, decreases embryonic development when IVF is performed 22 to 24 hpm (Edwards and Hansen, 1996; Lawrence et al., 2004; Payton et al., 2004; Edwards et al., 2005; Schrock et al., 2007) in a manner similar to cows that experienced elevated rectal temperatures > 41.0°C for the first 12 h of estrus (Putney et al., 1989). Agreement of in vivo and in vitro data showing susceptibility of oocytes to heat stress may be attributable to similarities in the microenvironment in which the oocyte is matured in. Specifically, both in vivo and in vitro-derived oocytes remain surrounded by interconnected cumulus cells during maturation. Moreover, it is interesting that pregnancy rates in Georgia

and Florida have been reported to be 25 to 35% in the winter and 10 to 15% in the summer (De Vries and Risco, 2005). It is important to note that these pregnancy rates are similar to blastocyst development reported for in vitro derived nonheat-stressed and heat-stressed oocytes, respectively (Lawrence et al., 2004; Schrock et al., 2007). Nonetheless, utilization of in vitro studies will allow for design of more concentrated in vivo studies utilizing fewer animals to study the effects of heat stress on the maturing oocyte.

### ***Direct Effects of Heat Stress on the Maturing Oocyte***

The maturing oocyte is particularly susceptible to direct application of heat stress as exposure to a physiologically-relevant elevated temperature in vitro decreases embryonic development when fertilization is performed 22 to 24 h after IVF (Edwards and Hansen, 1996; Lawrence et al., 2004; Roth and Hansen, 2004; Edwards et al., 2005; Schrock et al., 2007). Specifically, culturing oocytes at 41.0°C during the first 12 h of maturation generally results in a 42 to 65% decrease in proportion of oocytes that develop to the blastocyst stage (Lawrence et al., 2004; Roth and Hansen, 2004; Edwards et al., 2005; Schrock et al., 2007).

While little is known about the cause of decreased embryonic development of heat-stressed oocytes that are inseminated, altered fertilization rate is not likely a causative factor. Previous efforts in our laboratory have shown that exposure of maturing oocyte to an elevated temperature of 41.0°C for the first 12 h of maturation had no effect on the proportion of oocytes that are penetrated, monospermic, and undergo pronuclear formation at 6, 10, 13, and 18

formation (Schrock and Edwards, unpublished data). This is in agreement with Putney et al. (1989) who demonstrated a reduction in development without consequence on fertilization in heat-stressed heifers. These results are in contrast to those reported by Roth and Hansen (2005). In that study numbers were limiting as 46 control and 61 heat-stressed oocytes were evaluated for the proportion that were unfertilized and underwent pronuclear formation. In addition, alterations in carbon dioxide (**CO<sub>2</sub>**) concentration were confounded with heat stress.

Like fertilization rate, the direct effect of heat stress on the maturing oocyte does not necessarily decrease ability of presumptive zygotes to cleave after fertilization (Edwards and Hansen, 1996,1997; Lawrence et al., 2004; Edwards et al., 2005; Schrock et al., 2007) but it on occasion reduces the proportion of embryos that develop to the 8- to 16-cell stage (Lawrence et al., 2004). Reduced total cell number in blastocyst derived from oocytes heat stressed at 41.0 or 42.0°C has also been observed (Lawrence et al., 2004; Roth and Hansen, 2004; Ju et al., 2005) which may contribute to the poorer development of heat-stressed oocytes.

While it has been well demonstrated that heat stress reduces blastocyst development and that cleavage generally remains unaffected (Edwards and Hansen, 1996, 1997; Lawrence et al., 2004; Edwards et al., 2005; Schrock et al., 2007), little is known about the specific effects of heat stress on the maturing oocyte to alter the ability of resulting embryos to undergo further development (i.e., compaction). Since compaction is requisite for blastocyst formation



(Watson et al., 1990), additional effort is warranted to clarify effects of heat stress on the oocyte to possibly alter compaction. Doing so is of important in delineating the stage of development that embryos derived from heat-stressed and nonheat-stressed oocytes first diverge.

Negative consequences of heat stress during oocyte maturation to decrease development to the blastocyst stage after IVF may in part be mediated by heat-induced increases in apoptosis. Roth and Hansen (2004, 2005) demonstrated that more oocytes exposed to elevated temperature (41.0°C) during the first 12 h of maturation were TUNEL positive. Moreover, coincident with the increase in TUNEL positive heat-stressed oocytes, were higher levels of caspase activity than those cultured at 38.5°C (Roth and Hansen, 2004, 2005).

Reduced development may also be due in part to heat-induced perturbations occurring in the ooplasm. Specifically, Payton et al. (2003) reported an increase in GSH levels in oocytes exposed to heat stress which may be indicative of an increase in free radicals in heat-stressed oocytes. In support of this, addition of 5  $\mu$ M retinol to maturation medium of heat-stressed oocytes ameliorated negative effects of heat stress to reduce blastocyst development (Lawrence et al., 2004).

Decreased abundance of Poly(A) mRNA and protein synthesis may also contribute to reduced development of heat-stressed oocytes after IVF. While the abundance of total RNA remains unchanged, abundance of Poly(A) mRNA has been shown to decrease in oocytes cultured at 41.0°C for 12 h (Payton and Edwards, 2005). In addition, the culture of maturing oocytes with intact cumulus

cells at 41.0 or 42.0°C for the first 12 h of maturation resulted in a 28 and 45% reduction in total intracellular proteins synthesized, respectively (Edwards and Hansen, 1996). However, when oocytes were denuded of associated cumulus prior to culture at an elevated temperature, the decrease in protein synthesis was exacerbated (Edwards and Hansen, 1996) suggesting that cumulus cells may play a vital role in thermoprotection. In support of this, both de novo synthesis of total intracellular proteins (Edwards and Hansen, 1997) and heat shock protein **(HSP)** 70 (Edwards and Hansen, 1996) were not altered in oocytes cultured at 41.0 or 42.0°C when associated with their cumulus.

While cumulus cells may play a role in protecting the oocyte from some of the negative consequences of elevated temperature, it is unclear the extent to which they may be altered by heat stress. Lenz et al. (1983) reported that cumulus cells derived from oocytes matured at 41.0°C for the entire period of maturation (24 h) did not undergo expansion or synthesize hyaluronic acid to the same extent as nonheat-stressed controls. In addition, Lenz et al. (1983) reported a reduction in the number of heat-stressed oocytes that had a visible polar body suggesting an alteration in nuclear maturation. While a different study showed that oocyte maturation (progression to MII) was inhibited by exposure to 41.0°C (Roth and Hansen, 2005), extensive analysis in our laboratory has shown no effect to inhibit maturation. For example, similar proportions of control and heat-stressed oocytes progressed to MI and MII by 12 and 21 h of maturation. In this study, greater than 1,200 oocytes were examined using 205 to 239 oocytes

per treatment. Disparity in results may have been due to Roth and Hansen (2005) confounding alterations in CO<sub>2</sub> concentrations with 41.0°C treatment.

A major finding of our laboratory has been to show that heat-stressed oocytes mature at a faster rate than nonheat-stressed controls. Specifically, culture at 41.0°C for the first 12 h of maturation resulted in more heat-stressed oocytes progressing to MI and MII by 8 and 18 h, respectively. In addition, hastened cytoplasmic maturation has also been observed in heat-stressed oocytes (Edwards et al., 2005). Specifically, heat stress (41.0°C for 12 h) resulted in a decrease in the proportion of type II CG in heat-stressed oocytes which was coincident with an increase in type III CG determined at 24 h post maturation (**hpm**).

Taken together, these data demonstrate that the direct effects of heat stress are to hasten both nuclear and cytoplasmic maturation. The end consequence of this effect is most likely the fertilization of an “aged” oocyte if IVF is performed at the same time as controls (22 to 24 h after IVM). Because oocytes have a limited fertile lifespan of 4 to 10 h depending on whether cultured in vivo or in vitro (Hunter, 1985; Ward et al., 2002) fertilization of an aged oocyte is likely contributing to decreased development to the blastocyst stage observed in heat-stressed oocytes. In support of this, fertilizing heat-stressed oocytes 4 to 6 h earlier improved blastocyst development which approached values observed for nonheat-stressed oocytes fertilized at 24 hpm (Schrock et al., 2007).

## ***Effects of Heat Stress on the Preattachment Embryo***

Like the maturing oocyte, the early cleavage-stage embryo is susceptible to direct application of heat stress to reduce development. Effects of heat stress to reduce development are temperature, duration, and stage dependent. For example, an acute heat stress of bovine 2-cell embryos at 40.0°C for 1 h (Al-Katanani and Hansen, 2002) or 40.0°C for 3, 6, 9, or 12 h (Rivera and Hansen, 2001) did not have a negative effect on further development to blastocyst. However, when heat stress was applied to 2-cell embryos at a higher temperature (41.0°C for 9 or 12 h), development to the blastocyst stage was significantly reduced or inhibited altogether (Rivera and Hansen, 2001). Similarly, Ealy et al. (1995) showed that applying a heat stress of 40.0°C for 3 h to 2-cell embryos was not severe enough to affect subsequent development (29.2 and 31.4% developing past the 16 to 32-cell stage in heat-stressed and control 2-cell embryos, respectively); however, when culture temperatures were elevated to 41.0°C or 42.0°C for 3 h, the result was a significant reduction in subsequent development (10.6 and 2.6% for 41.0°C or 42.0°C, respectively). Moreover, Edwards and Hansen (1997) demonstrated that exposing 2-cell embryos to 41.0°C for 12 h decreased blastocyst development from approximately 50 to 8%.

While 2-cell embryos are particularly susceptible to heat stress (Ealy et al., 1993, 1995; Edwards and Hansen, 1997), it is important to note that as embryos proceed in development they gain the ability to withstand elevated temperatures (Ealy et al., 1993, 1995; Edwards et al., 1997). Specifically, compact morula and

blastocyst stage embryos are more thermotolerant to heat stress. This is true regardless if embryos are in vitro or in vivo derived (Ealy et al., 1993, 1995; Edwards and Hansen, 1997). For example, Ealy et al. (1993) heat stressed superovulated Holstein cows on days 1, 3, 5, or 7 after artificial insemination resulting in rectal temperatures that exceeded 41.0°C. Application of heat stress on day 1 after artificial insemination reduced the proportion of blastocyst recovered on day 8. By day 3 however, there was no effect of heat stress to decrease the proportion of blastocyst recovered (60, 34 and 49% blastocyst recovered from cows heat-stressed on day 3, day 1, or nonheat-stressed cows, respectively; Ealy et al., 1994). Interestingly, superovulated cows heat stressed on days 3, 5, and 7 after artificial insemination had higher blastocyst development than nonheat-stressed controls with 60%, 57%, 64%, and 49% developing to blastocyst, respectively (Ealy et al., 1993).

Responsiveness of in vitro derived compact morulae to heat stress parallels in vivo studies. Ealy et al. (1995) demonstrated similar blastocyst development when in vitro-derived compact morulae underwent heat stress at 41.0°C for 1 or 3 h (22.8% and 21.7%, respectively compared to 30.0% in nonheat-stressed controls). Similarly, Edwards and Hansen (1997) cultured compact morulae at 41.0°C for 12 h without a negative consequence on blastocyst development. In fact, 42% of heat-stressed compact morulae developed to the blastocyst stage versus 38% for nonheat-stressed controls (Edwards and Hansen, 1997). It was previously speculated that increased thermotolerance in compact morulae may be attributable to increases in cell

number or protective molecules such as HSP and GSH (Ealy et al., 1992; reviewed by Edwards et al., 2001)

## **Possible Heat-Induced Alterations in Sex Ratio of Preattachment Embryos**

Gender-specific differences in the ability to withstand elevated temperatures have been described (reviewed by Edwards et al., 2001 and by Gutiérrez-Adán et al., 2006). In particular, Kawarsky and King cultured 8 to 16-cell bovine embryos at 38.5 or 42.0°C for 0.5, 2, or 4 h (data reported in a review by Edwards et al., 2001) and showed that a higher percentage of embryos that developed to the blastocyst stage were females compared to nonheat-stressed embryos (58, 70, 60, and 51 for in embryos heat-stressed for 0.5, 2.0, 4.0 h, and nonheat-stressed oocytes, respectively). In agreement with this, Gutiérrez-Adán (2005) reported in an abstract that after the compact morula stage, a higher proportion of female embryos survive after exposure to heat stress either in vivo and in vitro. Possible increases in survivability of female embryos have also been suggested in murine embryos. Pérez-Crespo et al. (2005) reported that culture of murine compact morulae at 41.0°C for 24 h did not result in deviation from the expected 1:1 sex ratio at the blastocyst stage. However, when those embryos were transferred into surrogate recipients, a 51 versus 17% implantation rate of embryos derived from nonheat-stressed and heat-stressed oocytes resulted, respectively. However on day 14, 72% (28/39) of female embryos were recovered from surrogates receiving heat-stressed embryos

compared to 45% (37/82) receiving nonheat-stressed embryos (Pérez-Crespo et al., 2005).

Possible increases in survival of female embryos after exposure to a physiologically-relevant elevated temperature may be due to the fact that during early embryonic development, the female embryo has two active X-chromosomes. In the bovine embryo, X-inactivation is first evident at the early blastocyst stage and progressively increases up to the hatched blastocyst stage (De La Fuente et al., 1999). With two active X-chromosomes, the copies of sex-linked genes associated with the X-chromosomes such as glucose-6-phosphate dehydrogenase (**G6PD**) doubles; thereby, possibly increasing the survivability of female embryos (reviewed by Kochhar et al., 2001). In fact, female blastocysts show significantly higher G6PD mRNA expression (Gutiérrez-Adán et al., 2000) which may contribute to lower hydrogen peroxide levels (Pérez-Crespo et al., 2005) and possibly reduced levels of free radicals than their male counterparts. In support of this, Pérez-Crespo et al. (2005) found no difference in the resulting sex of murine compact morulae cultured at 41.0°C for 24 h in an inhibitor of G6PD.

In contrast to results observed with heat-stressed embryos, exposure of blastocyst-stage embryos to other stressors have been shown to increase the proportion of males in surviving blastocysts. Nedambale et al. (2004) reported 29 to 33% of male blastocysts survived 48 h postwarming after vitrification compared to only 14 to 18% of females. Interestingly, this pattern was not

observed in those blastocysts not exposed to vitrification (Nedambale et al., 2004).

***Effects of Heat Stress on the Maturing Oocyte to Possibly Carry Over into Preattachment Development to Increase Susceptibility of Compact Morulae to Heat Stress***

The Barker hypothesis states that “adverse influences early in development, and particularly during intrauterine life, can result in permanent changes in physiology and metabolism, which results in increased disease risk in adulthood” (De Boo and Harding, 2006). Low birth weight has been correlated with an increase in adult disease such as hypertension, diabetes, and stroke (reviewed by Barker, 2004). It is estimated that children born under 5.5 pounds have an increased risk compared to those who weighed 9.5 pounds at birth (Barker, 2004). Maternal undernutrition is a possible cause of reduced birth weight associated with several diseases. In fact, rat offspring resulting from dams that were fed 30% of ad libitum intake had higher mortality rates, decreased fetal weight, and decreased placental weight (Woodall et al., 1996). Additionally, when these offspring were 30, 48 and 56 weeks of age, they showed increased blood pressure compared to those who were not undernourished (Woodall et al., 1996).

Like maternal undernutrition, oocyte aging also has been reported to have negative consequences that may not be apparent until after birth. Specifically, the result of aging human oocytes has been linked to an increased incidence of



diseases such as diabetes mellitus, schizophrenia, as well as decreased fertility of resultant daughters (reviewed by Tarin et al., 2000). Offspring resulting from murine oocytes artificially inseminated at 22 h after gonadotropin-releasing hormone (**GnRH**) injection (i.e., aged for 10 h) showed an increase in offspring mortality compared to those that were inseminated at 13 h after GnRH injection (aged 1 h; 32.4% per litter versus 29.0% per litter in oocytes aged for 10 h and 1 h, respectively; Tarin et al., 2002). Moreover, lower body weights at weaning along with abnormalities such as bone protuberances, shorter tails, and syndactyly (fusion of digits together) occurred in offspring resulting from aged oocytes (Tarin et al., 2002).

Since a major consequence of heat stress on the maturing oocyte is to accelerate various aspects of oocyte maturation (Edwards et al., 2005; Schrock et al., 2007), heat-induced perturbations in developmentally-important processes in the oocyte likely has long lasting consequences. With this in mind, the working hypothesis of the research described herein is that direct effects of heat stress on the maturing oocyte likely perturb developmentally-important processes that carry over to later stage embryos to increase their susceptibility to heat stress. To test this hypothesis, blastocyst development of compact morulae derived from control or heat-stressed oocytes was evaluated after culture at 38.5°C or 41.0°C.

## Chapter 3

### Materials and Methods

#### ***Materials***

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise specified. Modified Tyrode's Albumin Lactate Pyruvate (**TALP**; HEPES-TALP, IVF-TALP, and Sperm-TALP (Parrish et al., 1988) was prepared in the laboratory. Potassium simplex optimized medium was prepared as described previously (Biggers et al., 2000), but modified to contain 0.5% BSA, 10 mM glycine, 1 mM L-glutamine, 1 x nonessential amino acids, 50 U/ml penicillin, and 50 µg/ml streptomycin (**mKSOM**). Medium-199 (**M199**), penicillin-streptomycin, and gentamicin were purchased from Invitrogen (Carlsbad, CA, USA). Follicle stimulating hormone (**FSH**) was obtained from Vetrepharm Canada Inc. (London, Ontario, Canada). Fetal bovine serum (**FBS**) was purchased from BioWhittaker (Walkersville, MD, USA). SYBR® Green Master Mix was purchased from Applied Biosystems, Inc. (Foster City, CA, USA). Frozen semen was generously provided by Ultimate Genetics, Inc. (Wheelock, TX, USA).

#### ***In Vitro Production of Embryos***

In vitro production of embryos was performed as previously described (Edwards et al., 2005; Schrock et al., 2007). Briefly, cumulus-oocyte complexes (**COCs**) were harvested from antral follicles (3 to 10 mm) from abattoir-derived

ovaries. Only those COCs with a dark, evenly granulated cytoplasm with a compact cumulus were cultured in groups of 45 to 50 for 24 h in 500  $\mu$ l oocyte maturation medium (M199 containing Earle's salts, 10% FBS, 50.0  $\mu$ g/ml gentamicin, 5.0  $\mu$ g/ml FSH, 0.2 mM Na pyruvate, and 2 mM L-glutamine) in 5.5% CO<sub>2</sub> humidified environment. Oocytes presumed mature underwent IVF at 24 h of in vitro maturation (hIVM) with Percoll-prepared frozen-thawed semen (500,000 motile sperm/ml). Putative zygotes (**PZ**) were denuded of cumulus cells and associated spermatozoa at 13 to 17 h after IVF by vortexing for 5 min in HEPES-TALP containing 0.3 mg/ml hyaluronidase and then cultured in groups of 25 to 35 PZ per well in mKSOM at 38.5°C, 5.5% CO<sub>2</sub>, 7.0% O<sub>2</sub> and 87.5% N<sub>2</sub> in a humidified environment.

### **Heat Induced Perturbations during Oocyte Maturation Carry Over to Increase Susceptibility of Compact Morulae to Heat Stress**

Cumulus-oocyte complexes were matured at 38.5°C (thermoneutral) or 41.0°C (heat-stressed during first 12 hIVM, 38.5°C thereafter; Figure 2). Oocytes presumed mature underwent IVF at 24 hIVM. The number of PZ recovered as well as those that had visibly lysed was recorded after denudment of cumulus and associated spermatozoa. Ability of PZ to cleave (determined by recording the number of 1-, 2-, 4-, and 8- to 16-cell embryos) was assessed at 69 to 72 h after IVF. Ability of embryos to undergo compaction was evaluated at 138 to 144 h after IVF. At that time, an evaluator uninformed of treatment origin assigned

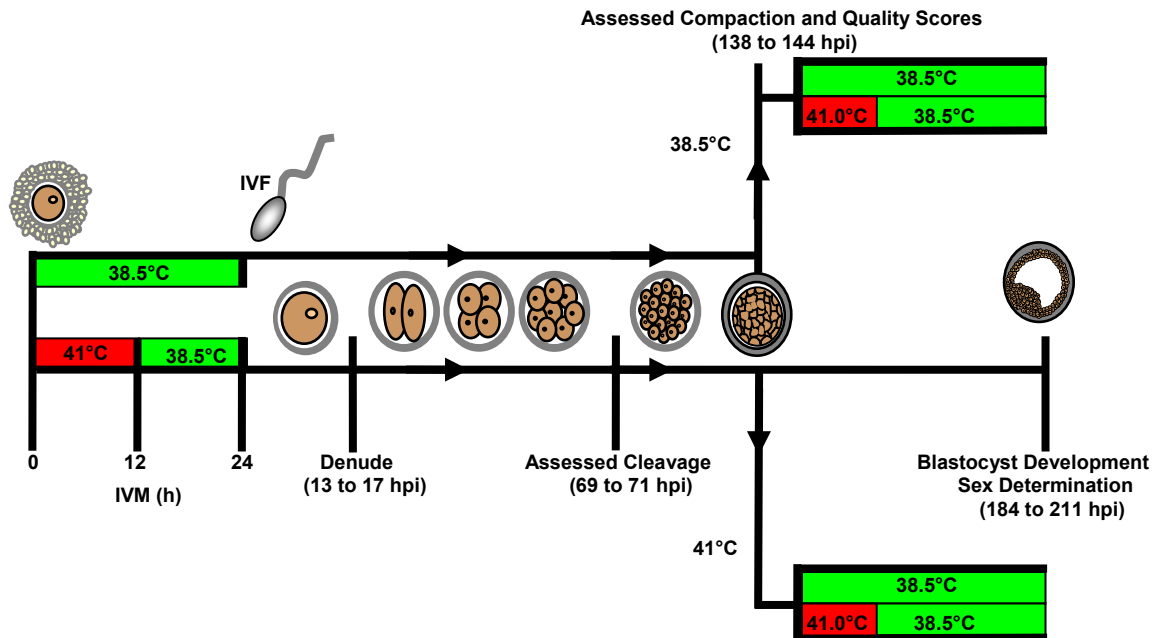


Figure 2. Experimental design and developmental endpoints. Cumulus-oocyte-complexes (COCs) underwent IVM at 38.5°C or 41.0°C. After a total of 24 h of IVM, COCs underwent IVF. At 13 to 17 hpi, putative zygotes (PZ) were denuded of cumulus cells and associated spermatozoa. Number of PZ recovered and those that had visibly lysed was recorded. Ability of PZ to cleave and compact was assessed at 69 to 71 and 138 to 144 hpi, respectively. Compacted morulae, kept separate according to IVM temperature, were assigned a quality score before culture at 38.5°C or 41.0°C for 12. At 184 to 211 hpi blastocyst stage and quality were recorded. In addition, sex of blastocyst-stage embryos was determined.

and recorded quality scores as previously described by Schrock et al. (2007) using IETS guidelines (Stringfellow and Seidel, 1998).

Compact morulae, kept separate according to origin (i.e., IVM at 38.5°C or 41.0°C), were then cultured in groups of 16 to 24 at 38.5°C or 41.0°C (12 h). Blastocyst development along with stage and quality score were assessed at 184 to 211 h after IVF by an evaluator uninformed of treatments. Also, the proportion of degenerate and embryos remaining at the compact morulae stage were also recorded.

### ***Sex Determination of Embryos***

Sex determination of blastocyst-stage embryos was performed using fluorescent-duplex PCR Bredbacka (2001). Fluorescent-duplex PCR was chosen over fluorescent in situ hybridization and electrophoretic approaches as this approach is simple and time efficient. This approach takes advantage of the fact that each amplified product melts (dissociates from double-stranded to single stranded) at a specific temperature based on amplicon sequence and length and that SYBR® Green I dye only associates with double-stranded DNA. The sex of each embryo can be determined from the dissociation curve performed following PCR cycles. Each reaction contained primers for Y and X chromosome-specific sequences to prevent experimental errors leading to false results due to absence of embryo template or failure of amplification reaction. A master mix containing uracil-DNA glycosylase (**UDG**; specifically cleaves previously amplified DNA) and

a hot start *Taq* was chosen to ensure that PCR carry-over contamination and non-specific product amplification were prevented, respectively.

Embryos were prepared for sexing by duplex PCR as described by Peippo et al. (2001) with the following modifications. To remove the zona pellucida (**ZP**) and accessory sperm embryos were placed in 0.5% pronase in Dulbecco's phosphate buffered saline and washed twice in HEPES-polyvinyl alcohol (**PVA**). HEPES-PVA was prepared the same as HEPES-TALP with the exception that 0.3% polyvinyl alcohol was substituted for BSA. Each washed embryo was transferred in 2  $\mu$ l volume directly into 10  $\mu$ l of lysis buffer (10 mM Tris pH 8.5, 0.15  $\mu$ g/ml proteinase K) and subjected to 55°C for 60 min for proteinase K digestion followed by 95°C for 10 min to inactivate proteinase K. Following lysis incubation, samples were either immediately subjected to PCR assay or stored at -20°C until time of amplification.

Sex determination of 2  $\mu$ L of embryo lysate was performed using Power SYBR® Green Master Mix in a final reaction volume of 25  $\mu$ l which contained 200 nM of forward and reverse primers for both SRY and XIST (Table 1). Amplification and melt curves were performed on the ABI 7300 Real-Time PCR system. Reaction conditions were 2 min at 50°C for AmpErase uracil-N-glycosylate activity and 10 min at 95°C for Amplitaq Gold activation, followed by 35 cycles of 15 sec at 95°C for melting, 30 sec at 60°C for annealing and 30 sec at 72°C for extension. Immediately following amplification cycles, samples were subjected to increasing temperatures (60 to 95°C). Fluorescent readings were

Table 1: Characteristics of Primers Used in Duplex PCR for the Detection of Bovine X and Y Chromosomes. Primers were designed with Primer 3 software (Open source).

Gene	Accession #	Sequence (5'-3')		Amplicon Location	Melt Temp (°C)
<b>SRY</b>	AF148462	Forward	ACCCGATGCATGTAGAGACATTG	1474-1496	78.4
		Reverse	GACTGTGACCGGGTTAATTGG	1553-1573	
<b>XIST</b>	AF104906	Forward	CAGGGGTGTTTGACCGTTAC	1543-1562	82.7
		Reverse	CCAATCCTGACTGGCTGAAT	1763-1783	

taken constantly during changes in temperature to monitor SYBR® Green dissociation from amplified fragments.

At the end of each duplex PCR, each sample's dissociation curve was examined. An embryo with two amplicon peaks was designated as a male; whereas, an embryo with one amplicon peak was designated as a female (Figure 3). Prior to performing experimental reactions, the identity of each amplicon product was confirmed by electrophoresis and DNA sequencing. In addition, genomic DNA was extracted from male and female-derived cells to generate standards which were included within each experimental replicate. In addition, each time duplex PCR was performed, reactions containing a no template control (**NTC**) were also included. This fluorescent duplex PCR approach was validated prior to this experiment using in vitro-derived embryos. In total, 681 embryos were subjected to the sexing assay (196 compact morulae and 485 blastocyst-stage embryos) which resulted in 54% and 46% male and female embryos, respectively. These results are similar to those results reported previously for in vitro-derived embryos using PCR and either non-electrophoretic or electrophoretic approaches (Gutiérrez-Adán et al., 1999; Hasler et al., 2002). Relevant for research described herein, sex of resulting blastocysts was determined on 9 of the total 20 different occasions yielding 127 to 134 blastocysts per treatment group.



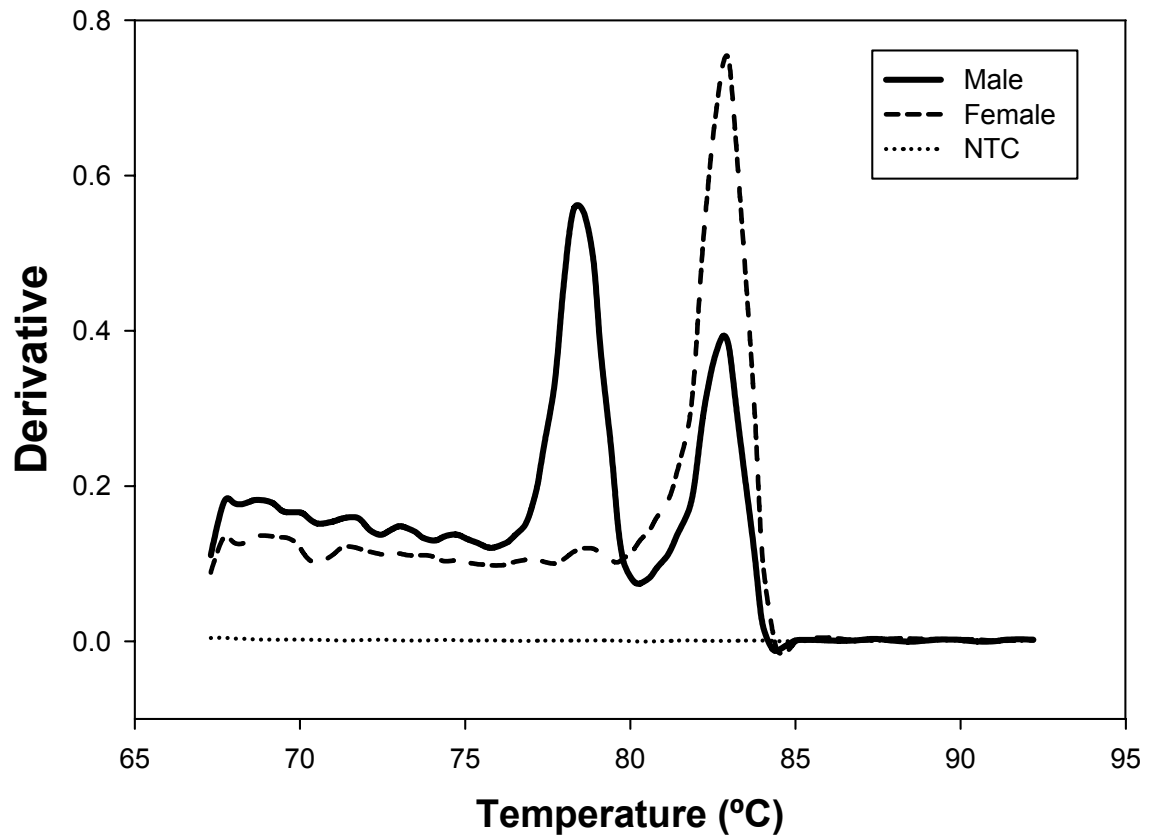


Figure 3. Dissociation peaks observed in male and female embryos and in no template controls (NTC). The Y axis is representative of the derivative or change in fluorescence and the X axis is the temperature at which the DNA melts (dissociates or transitions from double stranded to single stranded DNA).

## Statistical Analyses

To evaluate embryonic development after culture of maturing oocytes at 38.5 or 41.0°C, data were analyzed as a randomized block design, blocking on day of oocyte collection, using generalized linear mixed models (**PROC GLIMMIX**) in SAS (2005). GLIMMIX allows for analysis of fixed and random effects of both normally distributed (embryo quality) and binomial response data (all other variables). Fixed effects of temperature were evaluated. Treatment differences were determined using protected LSD and reported as least square means  $\pm$  SEM.

To evaluate the effects of heat stress during oocyte maturation to increase susceptibility of compact morulae to heat stress, data were analyzed as a randomized block design with a split plot using PROC GLIMMIX of SAS (2005). Orthogonal contrasts were performed to test preplanned comparisons. Treatment differences were reported as least square means  $\pm$  SEM using the inverse link option.

Because the quality of COCs from abattoir-derived ovaries may differ on a given day, developmental potential of control and heat-stressed oocytes was evaluated in retrospect. This was accomplished by pooling blastocyst development within a given replicate of compact morulae derived from oocyte matured at 38.5 or 41.0°C and then dividing by the number of PZ cultured. Doing so was important for three reasons: 1) it allowed for assessing the extent to which heat stress on the maturing oocyte decreases development after IVF, 2) it

provided an indirect assessment of the quality of starting material (i.e., COCs placed in culture to develop after IVF), and 3) it also allowed for a comparison of results to previous literature. When doing so, replicates with  $\geq 20\%$  blastocyst development for nonheat-stressed were designated as developmentally-competent; whereas those with blastocyst development less than 20% were deemed developmentally-challenged. In total, this experiment was replicated on 20 different occasions. On 5 different occasions, blastocyst development of nonheat-stressed controls ranged from 12.0 to 14.7%, warranting exclusion from primary analyses evaluating embryonic development after culture of oocytes at 38.5 or 41.0°C. To provide a more precise test of hypothesis (heat stress during maturation induces perturbations that carry over to increase susceptibility of resulting embryos to heat stress), only 13 out of the 15 experimental replicates whereby oocytes were deemed developmentally competent were utilized in analysis. These were experimental replicates whereby heat stress reduced blastocyst development. In total, 4,296 to 5,838 COCs were cultured at 38.5 or 41.0°C, respectively resulting in 454 to 538 compact morulae per treatment combination after IVF.

To examine the ability of heat stress on compact morulae to alter sex ratio of resulting embryos, data collected on 9 different occasions were analyzed as an RBD with a split plot using PROC GLIMMIX in SAS (2005). Treatment differences were reported as least square means  $\pm$  SEM using the inverse link option.

## **Chapter 4**

### **Results and Discussion**

Previous efforts have demonstrated that cleavage of embryos is not necessarily altered by application of heat stress during maturation but is coincident with heat-induced reductions in blastocyst development after IVF (Edwards and Hansen, 1996, 1997; Lawrence et al., 2004; Edwards et al., 2005; Schrock et al., 2007). To date, little progress has been made to identify the specific stages between cleavage and blastocyst development when embryos derived from heat-stressed oocytes diverge in developmental competence from nonheat-stressed controls.

In the current study, results showed that culture of COCs at 41.0°C did not reduce the proportion of PZ recovered or those that had visibly lysed after denudement of associated cumulus and spermatozoa. Furthermore, it was also noted that culture of COCs at 41.0°C did not alter ability of PZ derived from heat-stressed oocytes to cleave (Table 2;  $P = 0.19$ ). However, it was noted that the proportion of embryos that cleaved to the 8- to 16-cell stage was lower (Table 2;  $P = 0.01$ ) when a physiologically-relevant elevated temperature was applied during the first 12 h of maturation. This effect was coincident with an increase in the proportion of cleaved embryos that were at the 2-cell stage (Table 2;  $P = 0.05$ ). Similar findings were reported by Lawrence et al. (2004).

Specific factors whereby heat stress during the first 12 h of maturation “retarded” early cleavage divisions remain largely unknown. However, previous

Table 2. Developmental endpoints after performing IVF on COCs matured at 38.5 or 41.0°C.

IVM <sup>1</sup> Temp (°C)	Total COCs in OMM <sup>2</sup>	13 to 17 hpi <sup>6</sup>		69 to 71 hpi					138 to 144 hpi		
		PZ <sup>3</sup> Rcvd (%)	Lysed (%)	Clvd <sup>4</sup> (%)	1- cell (%)	2- cell* (%)	4- cell* (%)	8- to 16- cell* (%)	CM/PZ	CM/ Clvd	CM/ 8- to 16- cell
38.5	4,296	91.5	8.8	69.1	45.6	9.5 <sup>a</sup>	34.9	55.3 <sup>a</sup>	29.6 <sup>a</sup>	41.1 <sup>a</sup>	72.6 <sup>a</sup>
41.0	5,844	94.0	8.1	66.8	49.4	11.7 <sup>b</sup>	37.3	50.6 <sup>b</sup>	23.4 <sup>b</sup>	34.6 <sup>b</sup>	60.7 <sup>b</sup>
	SEM <sup>5</sup>	1.7	0.7	2.4	5.3	1.2	1.4	1.9	1.6	2.0	3.0
	P-Value	0.24	0.22	0.19	0.41	0.05	0.09	0.01	0.0001	0.02	0.01

Means within a column with different letters differ.

<sup>1</sup>IVM = in vitro maturation temperature.

<sup>2</sup>Cumulus oocyte complexes into oocyte maturation media.

<sup>3</sup>PZ = presumptive zygotes recovered.

<sup>4</sup>Clvd = cleaved.

<sup>5</sup>SEM = standard error of the mean.

<sup>6</sup>hpi = hours post insemination.

\*Proportion cleaved.

efforts of our laboratory showed that heat-stressed oocytes are fertilized (similar proportion was penetrated, monospermic, and underwent pronuclear formation at 6, 10, 13, and 18 hpi compared with nonheat-stressed controls; Edwards et al., unpublished data), minimizing concerns related to fertilization failure. In addition, previous efforts to Hoechst-stain cleavage-stage embryos derived from heat-stressed oocytes showed no increase in the proportion of fragmented embryos after IVF.

Instead, heat-induced reductions in development are likely in large part a consequence of fertilizing an “aged” oocyte. Previous efforts have demonstrated that a major effect of heat stress is to hasten developmentally-important processes during oocyte maturation (Edwards et al., 2005). An end result of this effect was to shift developmental responsiveness of heat-stressed oocytes 7.3 h earlier than nonheat-stressed controls. Although aging matured oocytes for 6 to 8 h prior to fertilization has been shown to perturb early cleavage divisions (Ward et al., 2002; Agung et al., 2006), it is important to note that early fertilization is not entirely effective for increasing development of heat-stressed oocytes to that of nonheat-stressed controls (Schrock et al., 2007). Because of this, other factors may also be problematic; possibilities include heat-induced alterations in the cytoskeleton (Roth and Hansen, 2005), chromosomal alignment, or spindle morphology (Tseng et al., 2004). Tseng et al. (2004) demonstrated that culture of oocytes at 41.5°C for 4 h after being matured for 24 h resulted in a 77% increase in abnormal chromosome alignment and spindle morphology compared to nonheat-stressed controls. Additionally, in *Trichogaster trichopterus* eggs

(thermoneutral temperature is 27.0°C), exposure to temperatures of 40.5 or 42.0°C blocked in mitosis (Bergan, 1960).

Ability of embryos derived from control or heat-stressed oocytes to undergo compaction was assessed at 138 to 141 hpi. Heat stress during the first 12 h of IVM reduced the proportion of 8- to 16-cell embryos that underwent compaction (Table 2;  $P = 0.01$ ). Reductions in compaction after heat-stressed oocytes underwent IVF at 24 hIVM were without consequence on morphological characteristics of resulting embryos. Quality scores of compact morulae derived from heat-stressed oocytes were similar to those derived from nonheat-stressed oocytes (2.1 and 2.1 for 38.5 and 41.0°C, respectively;  $P = 0.75$ ; SEM = 0.04).

The mechanism(s) through which heat stress during the first 12 h of IVM reduces proportion of 8- to 16-cell embryos that undergo compaction, remain unclear, but are likely multifactorial. During this time period in oocyte maturation, protein synthesis increases (Tomek et al., 2002; Coenen et al., 2004), transcriptional activity declines (Memili et al., 1998), and poly(A) mRNA (Payton and Edwards, 2005) abundance decreases rapidly. Reduced development after application of heat stress during this time has been coincident with an increase in GSH content (which may be suggestive of heat-induced increases in free radicals; Payton et al., 2003), decreases in protein synthesis (Edwards and Hansen, 1996, 1997) and reductions in abundance of maternal transcripts (Payton and Edwards, 2005). This latter effect is particularly concerning as the maturing oocyte is transcriptionally quiescent after GVBD thus any ability to compensate for any loss of transcripts is limited.

With this in mind, it is interesting to speculate that reduced ability of 8- to 16-cell embryos derived from heat-stressed oocytes to undergo compaction may be due to heat-induced reductions in maternal transcripts such as E-cadherin,  $\beta$ -catenin, or  $\alpha$ -catenin. Efforts of others have demonstrated the importance of these gene products for compaction by showing that microinjection of E-cadherin double stranded RNA into bovine zygotes decreased the proportion of embryos that undergo compaction (Nganvongpanit et al., 2006). In addition, it has been shown that culture of 4-cell murine embryos in the presence of both a protein synthesis inhibitor and E-cadherin antibody prevents compaction (Levy et al., 1986). The mRNA for E-cadherin and  $\beta$ -catenin are present in the maturing oocyte and during preattachment development (Barcroft et al., 1998; Ohsugi et al., 1999; Nganvongpanit et al., 2006).

The working hypothesis of the study described herein was that direct effects of heat stress perturbs developmentally-important processes during oocyte maturation that carry over to later stage embryos to increase their susceptibility to heat stress. To test this hypothesis, compact morulae derived from either control or heat-stressed oocytes were cultured at 38.5 or 41.0°C; blastocyst development was assessed thereafter. Effort to test this hypothesis is of importance as the dairy cow not only experiences heat stress during estrus but most likely experiences heat stress on consecutive days thereafter during summer.

Unlike the maturing oocyte, application of heat stress to compact morulae derived from nonheat-stressed oocytes was without a negative consequence on



blastocyst development (Figure 4; Panel A; orthogonal contrast of treatment a versus b;  $P = 0.91$ ; SEM = 3.1). Furthermore, stage (Figure 4; Panel A;  $P = 0.44$ ; SEM = 0.06) and quality (Figure 4; Panel A;  $P = 0.15$ ; SEM = 0.06) scores of resulting blastocysts were similar. While similar findings were reported previously (Ealy et al., 1993, 1995; Edwards and Hansen, 1997), inclusion of these treatment combinations in the experimental design was important to document the extent to which compact morulae in this study were thermotolerant.

As a next step, an orthogonal contrast was performed to evaluate blastocyst development of compact morulae derived from heat-stressed oocytes to those derived from oocytes never exposed to a heat stress. It was important to do so as developmental potential was previously not known. When this comparison was made, blastocyst development of compact morulae derived from heat-stressed oocytes was shown to be similar to those derived from nonheat-stressed controls (Figure 4; Panel A; orthogonal contrast of treatment c versus a and b;  $P = 0.26$ ; SEM = 3.1). Moreover, blastocyst stage (Figure 4; Panel A;  $P = 0.13$ ; SEM = 0.06) and quality (Figure 4; Panel A;  $P = 0.50$ ; SEM = 0.06) scores were similar regardless of treatment. These findings were significant as results demonstrated that if a heat-stressed oocyte was able to undergo compaction, it had the potential to develop further and at a comparable rate to those embryos derived from oocytes never exposed to heat stress. Taken together with previous results demonstrating reduced ability of 8- to 16-cell embryos derived from heat-stressed oocytes to undergo compaction, this study revealed that a.

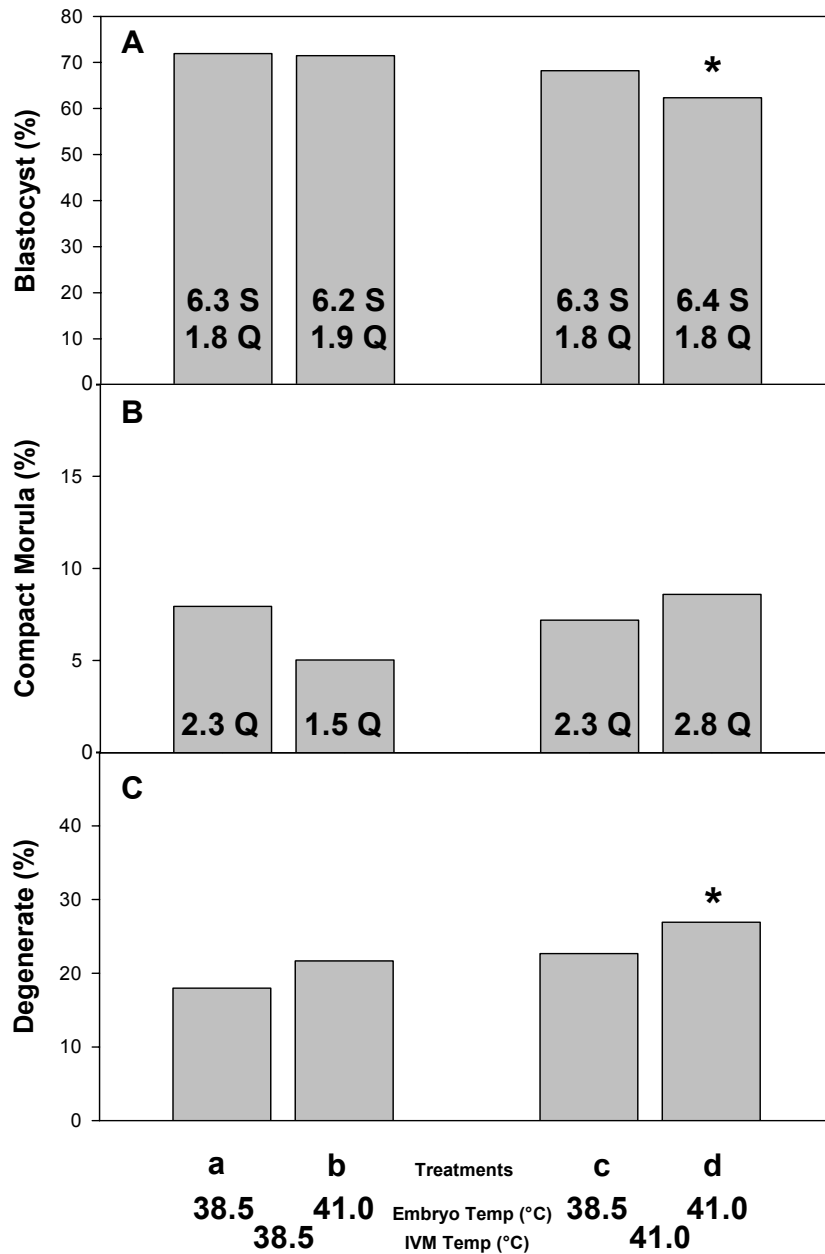


Figure 4. Blastocyst development of heat-stressed or nonheat-stressed compact morulae derived from COCs undergoing IVM at 38.5 or 41.0°C. Stage (S) and quality (Q) scores of blastocyst-stage embryos were included in individual bars for each treatment. For clarity, treatments were designated a, b, c, and d. The following orthogonal contrasts were significant: treatment d versus a, b and c ( $P = 0.007$ ; Panel A) for blastocyst development and treatment d versus a, b and c ( $P = 0.02$ ; Panel C) for proportion of degenerate embryos.

major divergence in developmental potential of heat-stressed oocytes occurs between the 8- to 16-cell and compact morulae stage.

In contrast, if compact morulae were derived from heat-stressed oocytes and then exposed to 41.0°C, ability to develop to the blastocyst stage was reduced (Figure 4; Panel A; orthogonal contrast of treatment d versus a, b and c;  $P = 0.007$ ; SEM = 3.1). This effect was coincident with an increase in the proportion of degenerate embryos (Figure 4; Panel C; orthogonal contrast of treatment d versus a, b and c;  $P = 0.02$ ; SEM = 3.3). Taken together, these observations provide strong supportive evidence for heat-induced perturbations in developmentally-important processes occurring during the first 12 h of maturation to carry over to the preattachment embryo to increase its susceptibility to heat stress. In the context of this experiment, compact morulae derived from heat-stressed oocytes were not as thermotolerant as those derived from oocytes that had not been exposed to heat stress.

While it is unclear what may be perturbed during maturation to increase the susceptibility of later stage embryos to heat stress, decreased blastocyst development may be suggestive of heat-induced perturbations in factors important for blastocoele formation. Important for blastocoele formation is the establishment of tight junctions (reviewed by Fleming et al., 2001) and  $\text{Na}^+\text{K}^+\text{ATPase}$  activity (Betts et al., 1997). Tight junctions are comprised of several proteins such as ZO-1 $\alpha$  and ZO-1  $\alpha$  (Fleming et al., 1989; Sheth et al., 1997), rab 13 (Sheth et al., 2000a), cingulin (Fleming et al., 1993), and occludin (Furuse et al., 1994). Efforts by Miller et al. (2003) demonstrated that ZO-1 $\alpha$ ,

ZO-1  $\alpha$ +, occludin and ZO-2 transcripts were present from the GV-stage oocyte to the blastocyst stage after fertilization. Transcripts for the  $\alpha$ 1 gene encoding the subunit of  $\text{Na}^+\text{K}^+\text{ATPase}$  have been shown to be present in the maturing bovine oocyte to blastocyst-stage embryo (De Sousa et al., 1998). Since previous studies evaluating expression of  $\alpha$ 2,  $\alpha$ 3, and  $\beta$ 2 genes encoding the subunit proteins of  $\text{Na}^+\text{K}^+\text{ATPase}$  reported similar expression patterns to that of  $\alpha$ 1, it is likely that these transcripts are also present within the maturing oocyte as well.

With the use of abattoir-derived oocytes, it is not uncommon to collect on a given day, oocytes that are otherwise “developmentally challenged” (i.e., poor blastocyst development after in vitro maturation, fertilization, and culture of resulting embryos). In the context of the study described herein, blastocyst development from oocytes collected on 5 different occasions ranged from 12.0 to 14.7%. Because developmental potential of oocytes clearly differed from what is expected (i.e.,  $\geq 20$  to 50% blastocyst development; Moore and Thatcher, 2006), exclusion from majority of analyses was warranted. However, as a continuum of our effort to clarify the extent to which developmental competence of the oocyte influences responsiveness of preattachment embryos to heat stress, these 5 replicates were utilized to examine blastocyst development of compact morulae derived from “developmentally challenged” oocytes. When this was done, it was observed that culture of compact morulae at 41.0°C reduced blastocyst development (Figure 5; Panel A; orthogonal contrasts of treatment a versus b;  $P = 0.01$ ; SEM = 5.0). Of those compact morulae that developed to blastocyst, stage (Figure 5;  $P = 0.82$ ; SEM = 0.1) and quality (Figure 5; Panel A;  $P = 0.18$ ;

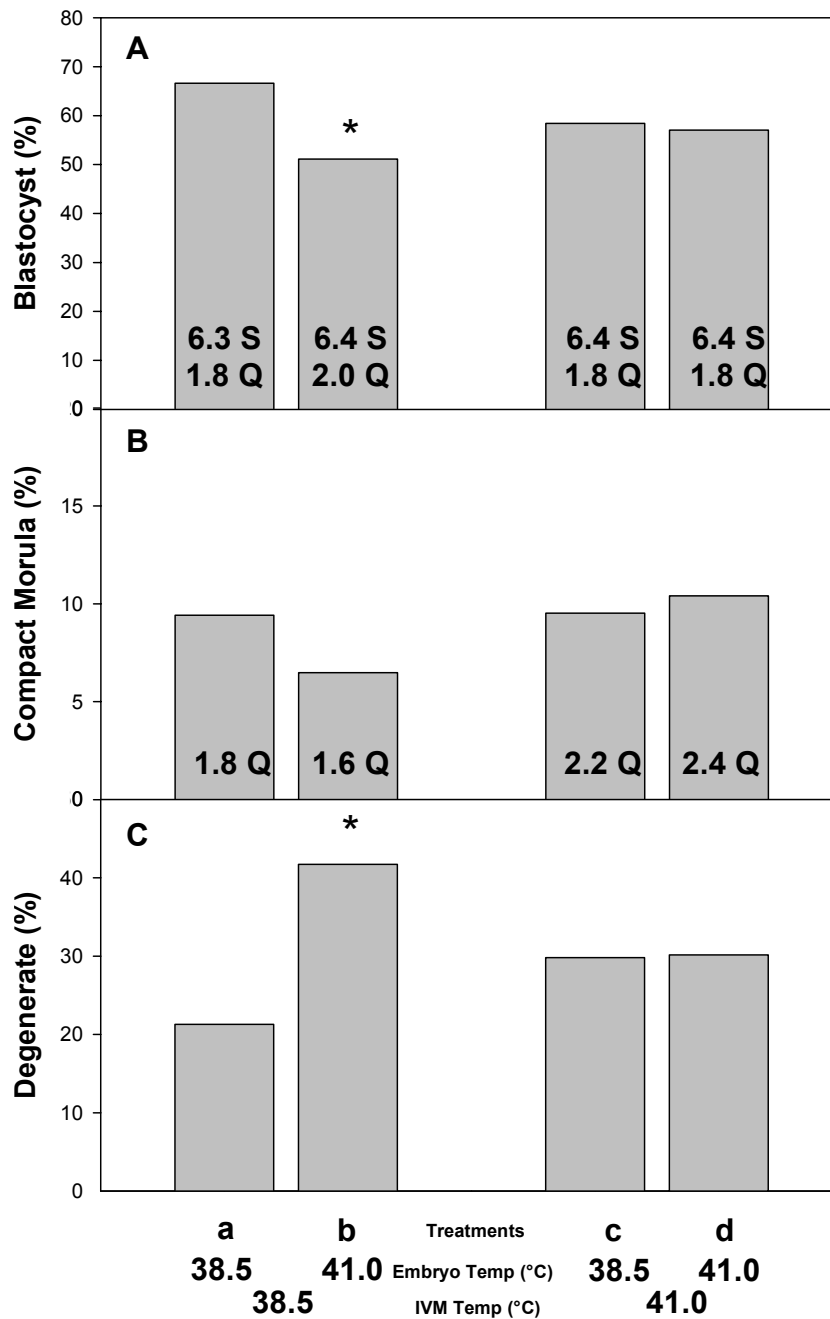


Figure 5. Blastocyst development of compact morulae derived from heat-stressed or nonheat-stressed but otherwise “developmentally challenged” oocytes following culture at 38.5 or 41.0°C. Stage and quality scores of blastocyst-stage embryos were included in individual bars for each treatment (Panel A). For clarity, treatments were a, b, c, and d. The following orthogonal contrasts were significant: treatment a versus b ( $P = 0.01$ ; Panel A) for blastocyst development and treatment a versus b ( $P = 0.001$ ; Panel C) for the proportion of degenerate embryos.

SEM = 0.1) scores were similar. This effect was coincident with an increase in the number of degenerate embryos (Figure 5; Panel C;  $P = 0.001$ ; SEM = 6.4).

The significance of this finding was to demonstrate that developmental potential of oocytes influences responsiveness of embryos resulting after IVF to stress. In the context of this experiment, using oocytes that were otherwise developmentally challenged resulted in compact morulae no longer being as thermotolerant as those derived from a pool of developmentally competent oocytes. Interestingly, this reduction in development was similar to that observed after compact morulae derived from heat-stressed oocytes were cultured at 41.0°C. Because not much is known regarding why abattoir-derived oocytes are sometimes developmentally challenged it is difficult to interpret these findings any further.

While it is possible that use of in vitro procedures may exacerbate negative effects of heat stress to induce perturbations in the oocyte that carry over to increase susceptibility of resulting embryos to heat stress, it is important to note that many aspects of in vivo and in vitro maturation parallel. In fact, both in vivo and in vitro derived oocytes undergo nuclear maturation at similar rates. Specifically, GVBD occurs between 4 to 8 h (in vitro-Hyttel et al., 1986b; Sirard et al., 1989; Edwards et al., 2005; in vivo-Kruip et al., 1983; Hyttel et al., 1986a). Thereafter, in vivo and in vitro-derived oocytes progress to MI and MII by 10 to 12 and 18 to 25 h, respectively and extrude their polar body by 16 to 21 h (in vitro-Hyttel et al., 1986b; Sirard et al., 1989; Edwards et al., 2005; in vivo-Kruip et al., 1983, Hyttel et al., 1986a). Translocation of cortical granules to the

oolemma is noted to occur between 20 and 30 h (in vitro-Hyttel et al., 1986 b; Edwards et al., 2005; in vivo-Kruip et al., 1983). Furthermore, the ability of invitro-derived embryos to support embryonic and fetal development to term has been clearly demonstrated. In fact, Hasler (2000) reported that in vitro-derived embryos established pregnancy and developed to term at comparable rates to in vivo-derived embryos.

It is also important to note the responsiveness of oocytes to heat stress is similar regardless if of in vivo or in vitro origin. In fact, oocytes cultured at 41.0°C for the first 12 h of maturation results in similar reductions in blastocyst development (Edwards and Hansen, 1996, 1997; Lawrence et al., 2004; Schrock et al., 2007) as those observed after Holstein heifers were exposed to a heat stress sufficient to raise rectal temperatures  $\geq 41.0^{\circ}\text{C}$  (Putney et al., 1989).

It is also with interest to note that pregnancy rates in Georgia and Florida are 25 to 35% in the winter compared to 10 to 15% in the summer (De Vries and Risco, 2005) which are comparable to blastocyst development observed in nonheat-stressed and heat-stressed oocytes cultured in vitro, respectively (Lawrence et al., 2004; Schrock et al., 2007). Nonetheless, similarity in responsiveness of maturing oocytes to heat stress regardless of in vivo versus in vitro derived origin are likely attributable to the fact that cumulus cells enclosing the oocyte create a microenvironment that is much like that found within the follicle.

Because of previous reports suggesting gender-specific differences in responsiveness of preattachment embryos to heat stress (reviewed by Edwards

et al., 2001), effort was also made to examine sex ratio of blastocysts that resulted after culture of compact morulae at 38.5 or 41.0°C. It is important to note that the temperature at which oocyte was matured did not influence sex ratio of resulting blastocysts. Rather, there was a strong tendency for culture of compact morulae at 41.0°C to increase the male to female ratio in surviving embryos that developed to the blastocyst stage ( $P < 0.06$ ; treatment means presented in Table 3). Male to female ratio of blastocyst-stage embryos of quality scores 1, 2, and 3 were 2.76, 1.33, and 0.90, respectively ( $P = 0.001$ ; SEM= 0.5).

These results were similar to others that have reported an increased survivability of males when compact morula-stage embryos were exposed to other stressors such as vitrification (Nedambale et al., 2004). However, they

Table 3. Male to female ratio of blastocyst-stage embryos

IVM <sup>1</sup> Temperature (°C)	Compact Morula Temperature (°C)	Total # Sexed	M/F <sup>3</sup> Ratio
38.5	38.5	127	1.07 <sup>B</sup>
38.5	41.0	134	1.59 <sup>AB</sup>
41.0	38.5	123	1.18 <sup>AB</sup>
41.0	41.0	130	2.23 <sup>A</sup>
			SEM <sup>2</sup> 0.4 P-Value 0.06

<sup>1</sup>IVM = in vitro maturation temperature.

<sup>2</sup>SEM = standard error of the mean.

<sup>3</sup>M/F = male to female sex ratio (male blastocysts / female blastocysts)



were in stark contrast to previous studies (Kawarsky and King, as published in Edwards et al., 2001; Pérez-Crespo et al., 2005) reporting an increase in the proportion of females that survived after application of heat stress at either the 8- to 16-cell stage or compact morulae. Differences in culture temperature, duration, and embryonic stage in which the heat stress was applied are apparent in different studies, therefore making it difficult to conclude why results among individual studies differed.

## **Conclusions/Implications**

Results described herein are significant as they not only clarify the extent to which heat stress during maturation reduces embryonic development after fertilization but are also informative of heat-induced perturbations that carry over to increase the susceptibility of resulting embryos to heat stress. These findings are important to agriculture as the dairy cow during summer months not only experiences heat stress during estrus but most likely experiences heat stress on consecutive days thereafter. With this in mind, results raise possible concerns about current practices of utilizing MOET in heat-stressed cattle with the intent of obtaining “developmentally-competent” embryos. Additionally, since it was also demonstrated that developmental competence of oocytes has an impact on the responsiveness of resulting embryos to heat stress, effort to clarify developmental competence of oocytes before utilizing in vitro derived embryos to improve fertility of heat-stressed dairy cows is needed.

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## **Vita**

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